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THIRD ANNUAL PROGRESS REPORT

TO

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

RADIOISOTOPIC BIOCHEMICAL PROBE FOR EXTRATERRESTRIAL LIFE

CONTRACT NO. NASr-10

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UNPUBLISHED PRELIMINARY DATA

March 30, 1964

RC#1

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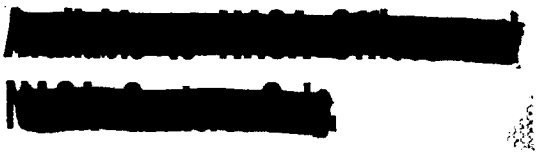

March 30, 1964

Gulliver III and Friend

Sheep Mountain, California

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I. SUMMARY

Gulliver III, the third model of the instrument designed to detect extraterrestrial life, has successfully detected terrestrial microbial life in twelve field tests this year. Four of the testing sites, specifically selected for their severe, adverse, environmental conditions, were: Sheep Mountain in the White Mountain Range of California on cold, rocky, barren terrain at an altitude of 12,000 feet; Death Valley, California which provided an arid, sandy environment; the salt encrusted desert flats of the Salton Sea in California; and Orange, Virginia, with its hard, iron-rich, clay surface. All of these extreme natural environment tests yielded good, positive responses in relatively short time periods. The remaining eight field trials, conducted locally, elicited positive responses. The Bard Parker antimetabolite was effective in inhibiting growth in the Gulliver control units associated with each test.

In situ determinations performed at the California field sites, in which labeled medium was applied directly to the soil, demonstrated considerably enhanced sensitivity. This suggested the feasibility of an alternative method of detecting metabolic gas evolution directly from microorganisms in their own habitation.

Medium development studies produced medium M9, a basal salts medium fortified with soil extract, and containing the organic C^{14} substrates - formate, glucose, lactate, and glycine. The formulation of this medium involved numerous evaluation studies of various labeled and unlabeled nutrients. The labeled nutrients tested included four of the

Miller Compounds, part of the general program of investigating the relevant Miller Compounds as labeled substrates. The effects of soil, beef, and yeast extracts; Panmede; sodium succinate and pyruvate; peptone, tryptone and additional nitrogen sources were investigated as well as various concentrations of labeled and unlabeled glucose. Other labeled C^{14} substrates examined were DL-α-alanine-1, glycine-1, DL-sodium lactate-1, DL-malic acid-3, nitrilotriacetic acid (labeled in the methylene groups), DL-tyrosine-1, and urea. One S^{35} labeled compound, sodium thiosulfate, was investigated. The M9 medium produced by these studies is currently in a process of refinement.

Increased specific activities of the standard C^{14} substrates, formate and glucose, and their resultant molar and radioactive levels were investigated in an effort to attain maximum sensitivity. Sodium formate at a higher specific activity, 25 mc/mM, proved to be more advantageous than the 5 mc/mM activity normally in use. The incorporation of the higher activity also permitted the use of a lower molar concentration. Surprisingly, increasing the specific activity of the glucose did not offer any advantage when used with the double or triple labeled medium.

As a result of the various studies mentioned above, the medium in present use is M9, containing the following constituents:

Unlabeled Nutrients % (w/v)		Labeled Nutrients % (w/v)	
K_2HPO_4	0.100	Sodium formate	0.002
KNO_3	0.050	D-glucose-U.L.	0.005
$MgSO_4 \cdot 7H_2O$	0.020	DL-sodium lactate-1	0.002
NaCl	0.010	Glycine	0.002
Soil Extract	100 ml/l		

A collection of specially selected pure cultures of micro-organisms, soils, and soil isolates has provided a wide range of test material for use as inocula in laboratory determinations. Positive responses have been obtained from all of the organisms and soils tested.

Of particular interest have been the responses of Thiobacillus thiooxidans and Thiobacillus novellus; Lactobacillus plantarum; Rhodospirillum rubrum; and the iron bacteria. The autotrophs produced equally good,positive responses from the C^{14} labeled M9 and a C^{14}, S^{35} labeled M9. A poor response was obtained with M9 medium containing only the S^{35} labeled substrate. The L. plantarum, a representative of a group of bacteria which are reported to produce little or no carbon dioxide from sugar fermentation, utilized the organically simple M9 medium as well as the nutritionally rich M5 medium to produce responses indicative of metabolism but not of growth. R. rubrum, a most versatile organism, has produced positive responses under photosynthetic, non-photosynthetic, aerobic and anaerobic conditions. Planchet determinations performed on four strains of iron bacteria (Leptothrix and Sphaerotilus species) cultured in the M9-formate-glucose-lactate-glycine medium have yielded definite growth responses.

Twelve soils, taken from diverse geographical areas in the United States, have formed the basis of the mixed culture studies. Pebbles; sand; high saline content, iron-rich, field and garden soils; have produced various degrees of responses depending upon the total numbers and types of organisms present, and also upon the complexity of the culturing medium. The M9 medium containing the four labeled substrates has consistently yielded the most sensitive responses.

In an effort to determine the source or sources of the general metabolic response produced by the mixed population of soil, sixty-four

individual isolates were cultured from five dissimilar soils. An initial, incomplete, comparative evaluation of twelve isolates revealed that two organisms were definitely common to at least two of the soils. Another pair of isolates appeared to be identical also. The twelve isolates produced a response range of 1×10^{-4} cpm/initial cell to 1.2 cpm/initial cell at a three hour incubation-collection period.

An anaerobic cabinet was designed and installed in the laboratory permitting twelve cultures to be monitored simultaneously under strictly anaerobic conditions. The results are automatically recorded. Positive responses have been obtained from Clostridium sporogenes, Clostridium perfringens, and Clostridium pasteurianum. The earliest growth response was produced in one hour by a seven day culture of C. perfringens inoculated into labeled medium.

Acrolein, argyrol, and mercuric chloride were screened for use as antimetabolites and were found to be unsuitable. Bard Parker germicide (isopropanol, methanol, formaldehyde and hexachlorophene) tested previously, continues to be the most effective inhibitor of metabolism.

Photosynthetic determinations on Chlorella pyrenoidosa substantiated the feasibility of using Gulliver to detect photosynthetic metabolic responses. A urea salts medium containing 2×10^{-3} M (10 uc/ml) DL-sodium lactate-1-C^{14} was found to be a suitable culturing medium. Initial responses to light change were more pronounced when the inoculated chambers were started in the light. A Mark III instrument has been modified for photosynthesis detection by placing the light source directly in the culturing chamber. The unit is being tested presently.

II. FIELD TESTS

Figure II-1 GULLIVER III FIELD TEST SITES

II. FIELD TESTS

An extensive and successful field testing program has been carried out with Mark III Gulliver. The primary objectives of the field trials were to investigate instrument reliability and biological response under normal or adverse conditions.

Twelve field trials were conducted during the year: eight locally and four in areas selected for specific environmental conditions. In all of the tests, two complete Gulliver units were operated simultaneously and automatically by an industrial programmer. A manually operated mock-up unit was added to the last four trials to serve as an uninoculated sterile control. Each unit contained a 3.0 ml ampoule of the C^{14} medium as well as a 0.5 ml ampoule of Bard Parker antimetabolite, thereby permitting selection of the instrument to receive the inhibitor. All mechanical operations were standardized and are discussed in Section IV, Instrumentation.

A. LOCAL FIELD TESTS

The Washington area field trials were conducted primarily for general evaluation of the instrument, of the testing procedures, and of the laboratory-developed medium.

A field adjacent to American Machine and Foundry Company, Alexandria, Virginia, provided the site for the majority of the tests. Ground conditions were predominantly dry and dusty, providing considerable soil for collection in all but one test in which a relatively small sample was retrieved from moist, solidly packed earth. The weather conditions were generally ideal: sunny, slight winds, with air temperatures ranging from $18^{o}C$ to $33^{o}C$. Medium M8-C^{14} (Table II-3) developed specifically

for soil microorganisms was employed throughout. A brief description of each test is given below. Details are presented in Figures II-2 to II-9.

Field Test - May 9 - Figure II-2

A routine field trial was conducted. The antimetabolite was injected into the unit first registering a metabolic response of 1000 cpm. Both responses were satisfactory, although the uninhibited response was lower than usual.

Field Test - May 16 - Figure II-3

The second test, conducted in the usual manner, produced a moderate response. The antimetabolite, injected at a 500 cpm response time, was effective.

Field Test - May 23 - Figure II-4

The antimetabolite was withheld from both instruments in order to establish the degree of replication present in the dual units. Considering the probable variance in the amount of soil sample collected, the responses were fairly consistent with one another.

Field Test - June 4 - Figure II-5

The inoculated uninhibited unit, using M8-C^{14}, produced an excellent response, which, when compared to the sterile control, illustrated definite evidence of growth although the control was higher than desired.

Field Test - June 19 - Figure II-6

A lower oxygen level was obtained in both culture chambers to favor the growth of anaerobic microorganisms (the Martian atmosphere most probably would support this type of bacteria). This was accomplished by leaving the baffle pin in place to prevent the inflow of air. Usually it is removed, permitting the establishment of atmospheric conditions

within the culture chamber through the open port. The response obtained was initially slower and lower than usual, time being required to deplete the oxygen initially present in the chamber before a suitable anaerobic atmosphere could be developed. The presence of anaerobic growth in the unit was subsequently verified in the laboratory. Segments of the inoculated retrieval line were asceptically removed from the unit, cultured in a liquid anaerobic medium (Difco Cooked Meat Medium), reinoculated into the labeled field test medium, and incubated aerobically and anaerobically (Brewer Jar). Monitoring of the resultant $C^{14}O_2$ produced 95 cpm from the aerobic test, 1155 cpm from the anaerobic test; thus verifying the presence of an anaerobic microflora in the field test chamber.

Field Test - June 27 - Figure II-7

The field demonstration was presented as part of an overall NASA review of extraterrestrial life detection experiments. The response was rapid and injection of the antimetabolite quickly separated the two curves.

Field Tests - July 2, July 17 - Figures II-8, II-9

In the last of the local field trials, the antimetabolite was injected immediately following the breaking of the medium ampoule. This permitted the inhibitor to become dispersed in the inoculated medium by the still revolving retrieval motor, thus inhibiting metabolism of the soil organisms from the onset. The desired inhibition appeared earlier and was more pronounced than in the previous controls.

- - -

It is evident from the accumulated data that the local field testing program was successful. The biological responses were all positive, the antimetabolite was effective, and the instrument performed

well. Areas requiring further research were indicated ($C^{14}O_2$ collector saturation, faulty projectile winding, and other minor mechanical problems) and emphasis was placed upon them in preparation for the extreme environmental field trials.

Figure II-2 FIELD TEST - GULLIVER III

Date: May 9, 1963 Weather: 27°C, sunny, slight wind

Location: Field - AMF, Alexandria, Va. Orientation: Detector up

Ground Condition: Dry, dusty Soil Sample Collected: Very good

Medium: 3 ml, M8, 6.16 uc/ml Antimetabolite: 0.5 ml, Bard Parker

Radioactive Substrates:	Sp. Act. (mc/mM)	uc/ml	mM/1	%(w/v)
Sodium formate	5.0	3.30	0.66	0.004
D-glucose-U.L.	3.0	0.66	0.22	0.004
DL-sodium lactate-1	5.0	2.20	0.44	0.005

Mechanical Function:

(a) Sequencing: No problems
(b) Projectiles: No problems
(c) Thermostat: Functioning

Components:

(a) Detector: Geiger Müller tube
(b) Collector: Gum coated, $Ba(OH)_2$

General Evaluation:

Mechanical operation was satisfactory although the decline in the cumulative count for the inhibited sample cannot be explained. Low response may have been due to saturation of collector. Antimetabolite appeared to be effective. However, initial response from uninhibited test may be result of smaller inoculum.

Response:

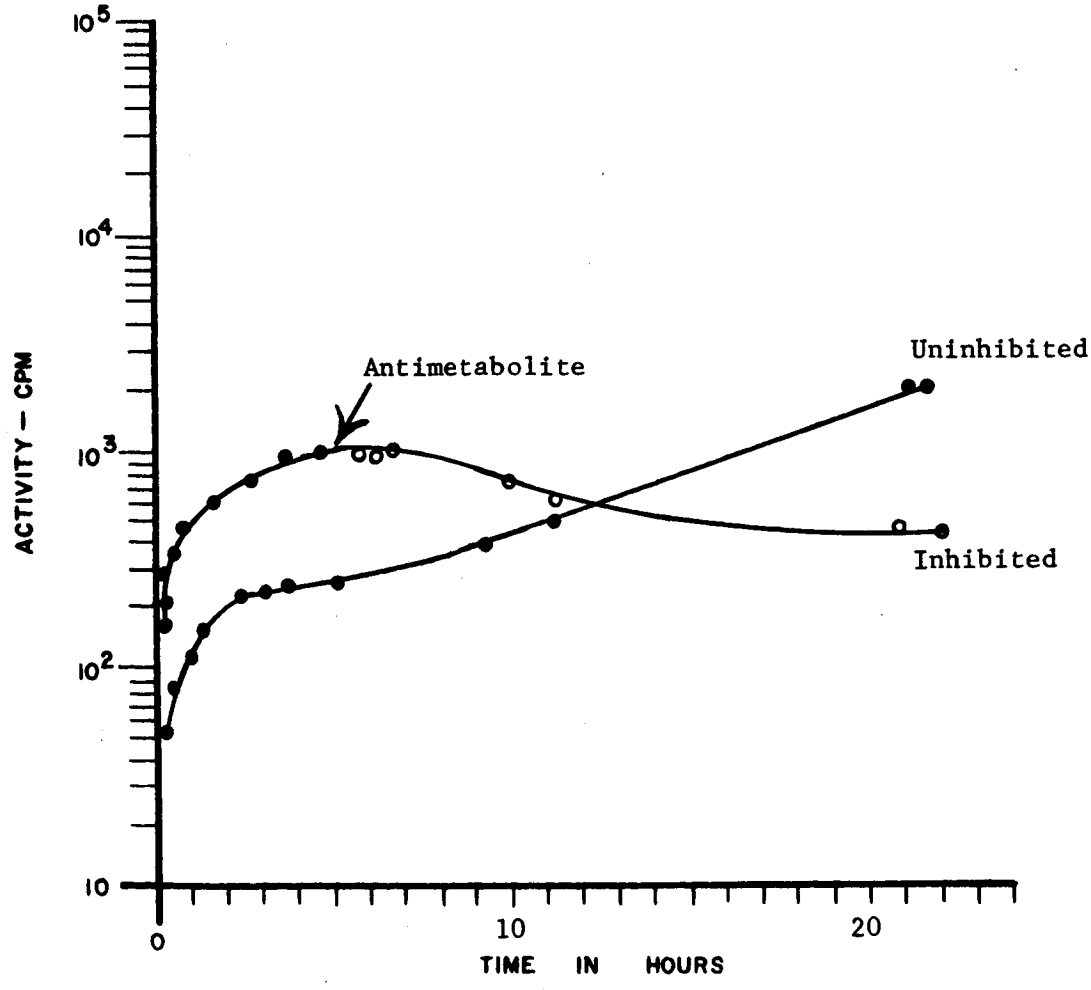


Figure II-3 FIELD TEST - GULLIVER III

Date: May 16, 1963

Location: Field - AMF, Alexandria, Va.

Ground Condition: Dry, not very dusty.

Medium: 3 ml, M8, 6.16 uc/ml

Weather: 18°C, overcast, slight wind

Orientation: Detector up

Soil Sample Collected: Good

Antimetabolite: 0.5, Bard Parker

Radioactive Substrates:

	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
Sodium formate	5.0	3.30	0.66	0.004
D-glucose-U.L.	3.0	0.66	0.22	0.004
DL-sodium lactate-1	5.0	2.20	0.44	0.005

Mechanical Function:

(a) Sequencing: No problems
(b) Projectiles: No problems
(c) Thermostat: Functioning

Components:

(a) Detector: Geiger Müller tube
(b) Collector: Gum coated, $Ba(OH)_2$

General Evaluation:

Satisfactory, although collectors may have become saturated. Responses were moderate. The antimetabolite was effective.

Response:

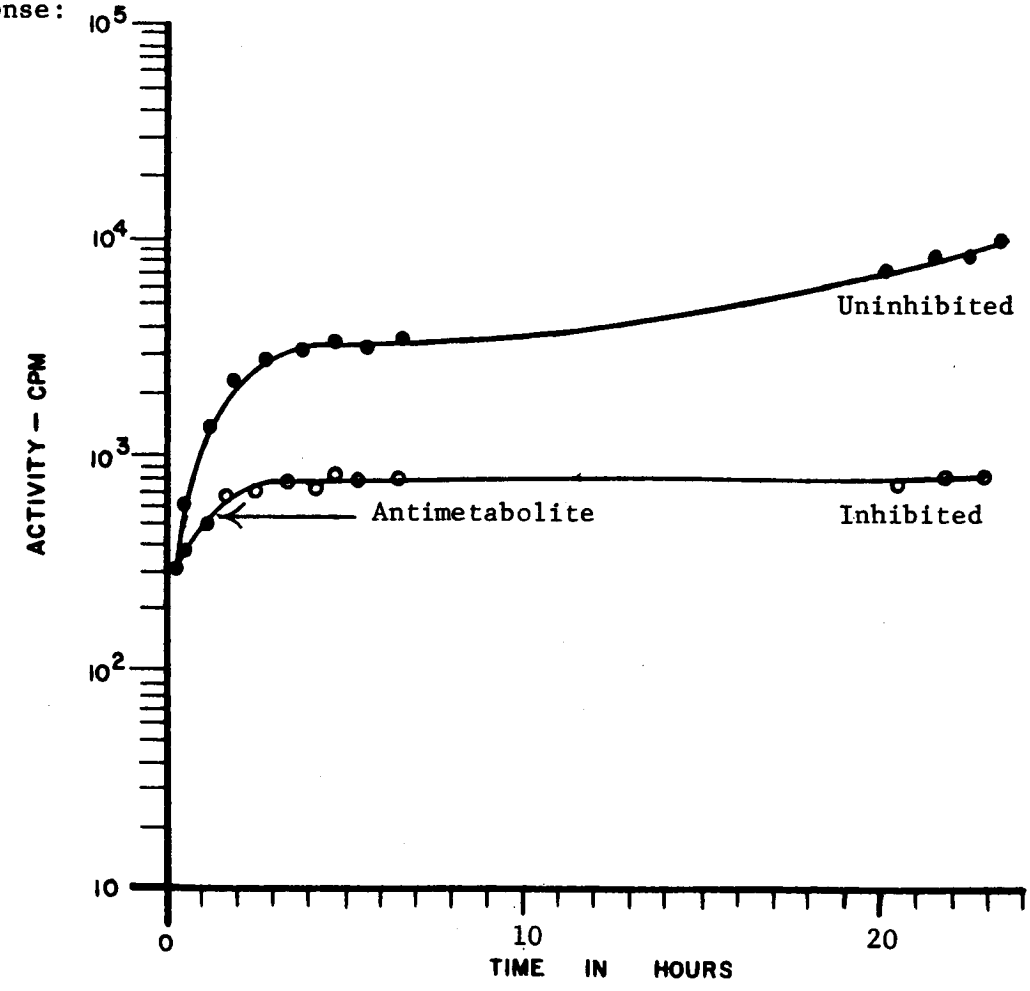


Figure II-5

FIELD TEST - GULLIVER III

Date: June 4, 1963

Weather: 22°C, sunny, slight wind

Location: Field - AMF, Alexandria, Va.

Orientation: Detector up

Ground Condition: Moist, packed soil

Soil Sample Collected: Fair

Medium: 3 ml, M8, 6.16 uc/ml

Antimetabolite: None used

Radioactive Substrates:	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
Sodium formate	25.0	3.30	0.13	0.001
D-glucose-U.L.	4.7	0.66	0.14	0.003
DL-sodium lactate-1	5.0	2.20	0.44	0.005

Mechanical Function:

(a) Sequencing: No problems
(b) Projectiles: One projectile remained attached, released at port
(c) Thermostat: Functioning

Components:

(a) Detector: Geiger Müller tube
(b) Collector: Gum Coated, $Ba(OH)_2$

General Evaluation: Antimetabolite was not used. One instrument inoculated, one was used as sterile control. Inoculated, uninhibited response was good.

Response:

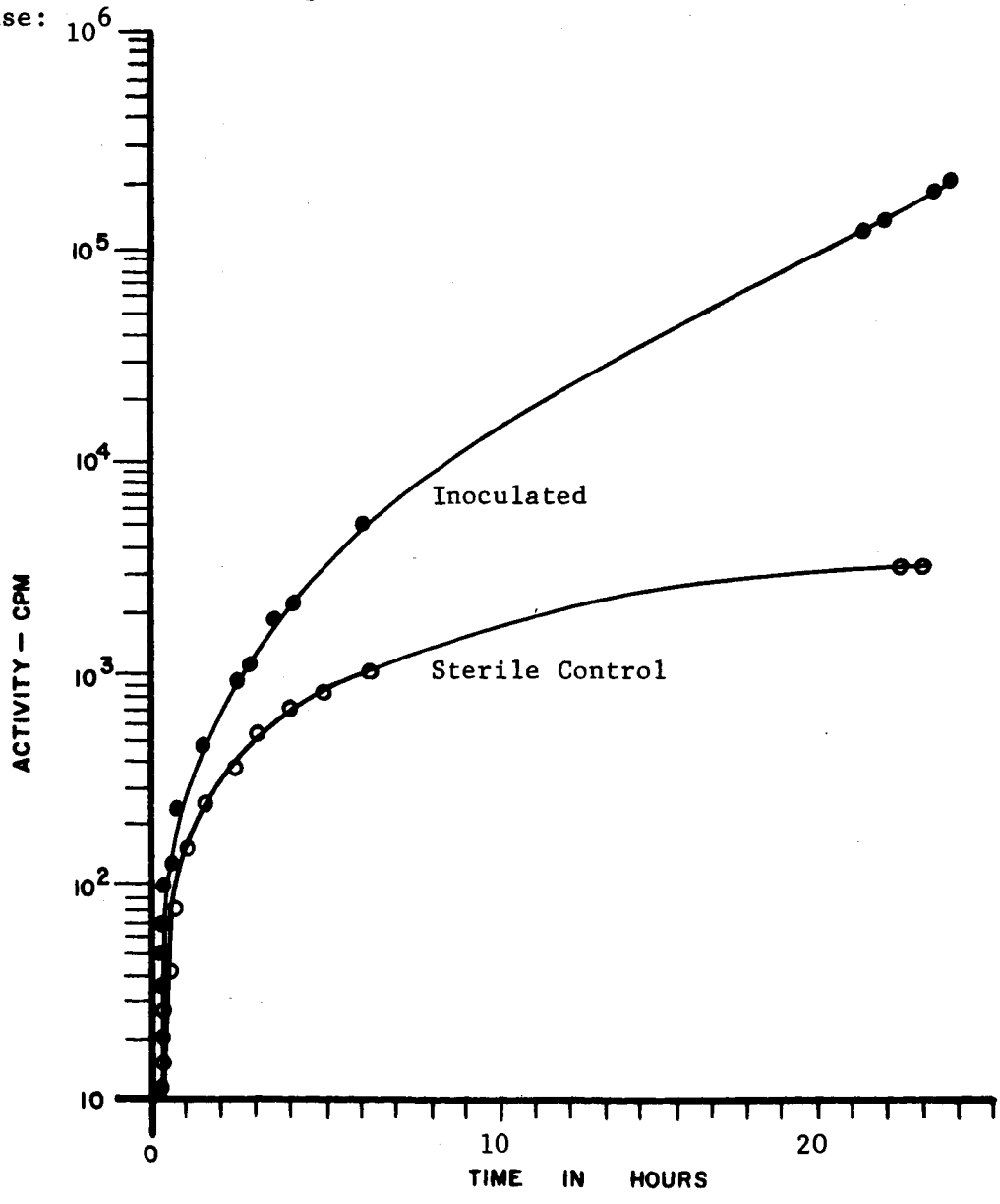


Figure II-6

FIELD TEST - GULLIVER III

Date: June 19, 1963

Location: Upshur Recreation Area, Washington, D. C.

Ground Condition: Dry, dusty

Medium: 3 ml, M8, 6.16 uc/ml

Weather: 29°C, sunny, slight wind

Orientation: Dectector up

Soil Sample Collected: Very good

Antimetabolite: 0.5 ml, Bard Parker

Radioactive Substrates:	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
Sodium formate	25.0	3.30	0.13	0.001
D-glucose-U.L.	12.0	0.66	0.06	0.001
DL-sodium lactate-1	5.0	2.20	0.44	0.005

Mechanical Function:

(a) Sequencing: No problems

(b) Projectiles: No problems

(c) Thermostat: Functioning

Components:

(a) Detector: Geiger Müller tube

(b) Collector: Krylon, $Ba(OH)_2$

General Evaluation: Mechanical operation was satisfactory. Low response was due to anaerobiosis and saturated collector. The antimetabolite was effective.

Response:

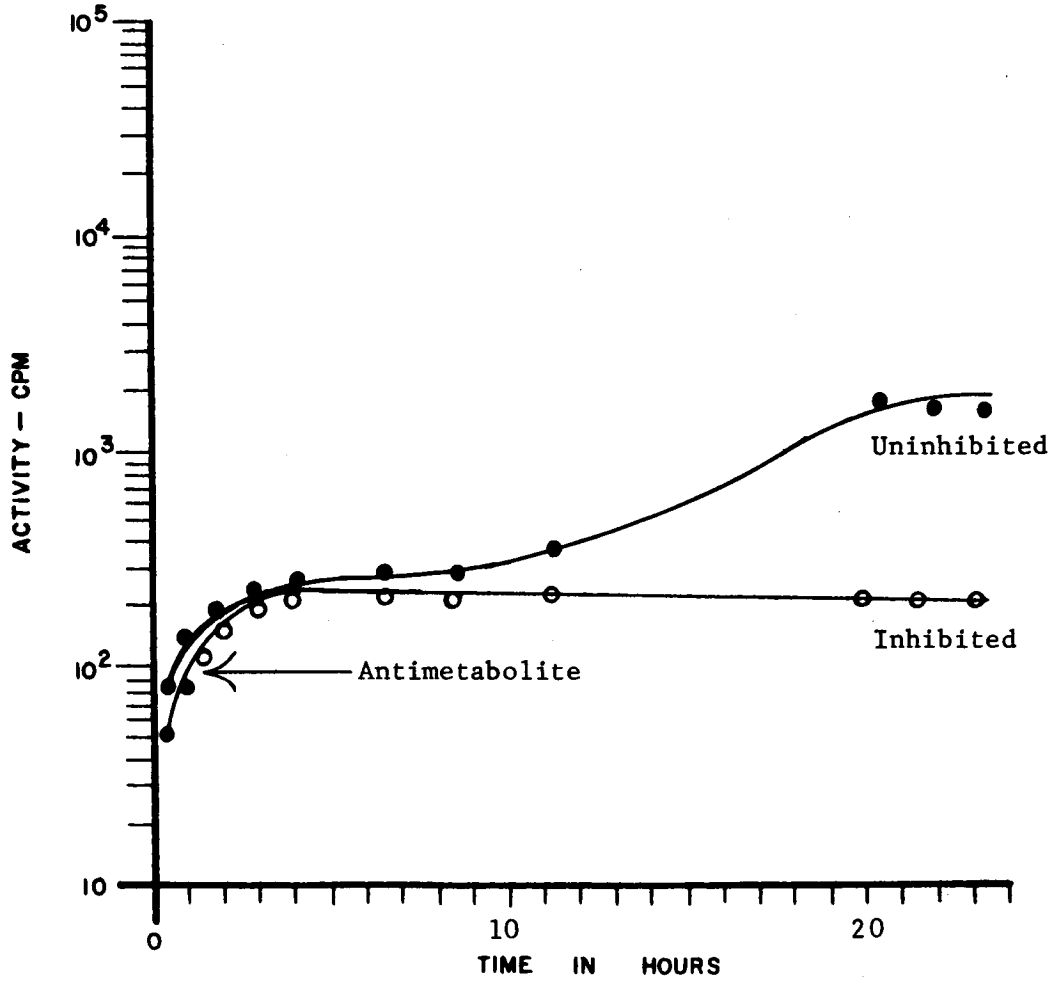


Figure II-7

FIELD TEST - GULLIVER III

Date: June 27, 1963

Location: Upshur Recreation Area, Washington, C. C.

Ground Condition: Dry, dusty

Medium: 3 ml, M8, 6.16 uc/ml

Weather: 30°C, hot, sunny, no wind

Orientation: Detector up

Soil Sample Collected: Very good

Antimetabolite: 0.5 ml Bard Parker

Radioactive Substrates:

	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
Sodium formate	25.0	3.30	0.13	0.001
D-glucose-U. L.	4.7	0.66	0.14	0.003
DL-sodium lactate-1	5.0	2.20	0.44	0.005

Mechanical Function:

(a) Sequencing: No problems
(b) Projectiles: No problems
(c) Thermostat: Functioning

Components:

(a) Detector: Geiger Müller tube
(b) Collector: Krylon, $Ba(OH)_2$

General Evaluation: Satisfactory, although collectors were saturated.

Response:

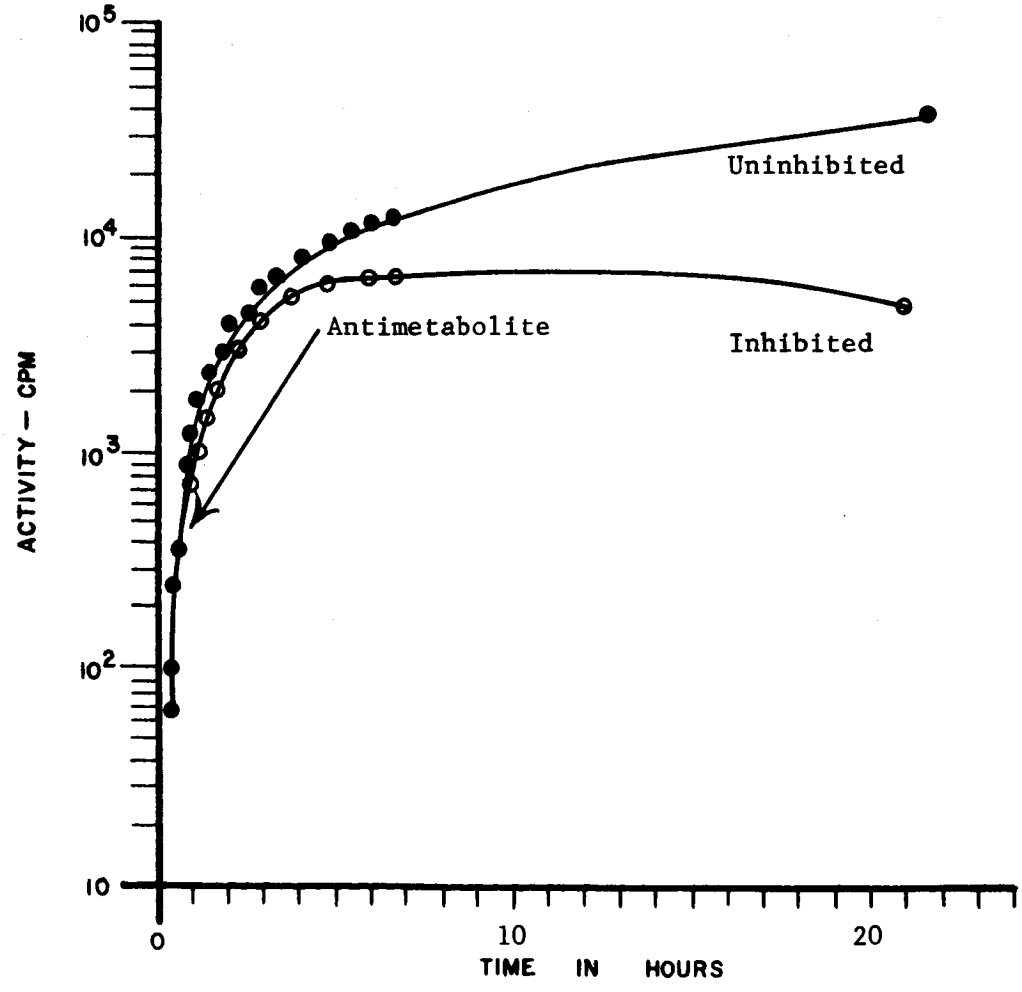


Figure II-8 **FIELD TEST - GULLIVER III**

Date: July 2, 1963 Weather: 33°C, sunny, slight wind

Location: Field - AMF, Alexandria, Va. Orientation: Detector up

Ground Condition: Dry, dusty Soil Sample Collected: Very good

Medium: 3 ml, M8, 6.16 uc/ml Antimetabolite: 0.5 ml, Bard Parker injected at time zero.

Radioactive Substrates:

	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
Sodium formate	25.0	2.30	0.13	0.001
D-glucose-U.L.	4.7	0.66	0.14	0.003
DL-sodium lactate-1	5.0	2.20	0.44	0.005

Mechanical Function:
(a) Sequencing: No problems
(b) Projectiles: No problems
(c) Thermostat: Not connected

Components:
(a) Detector: Geiger Müller tube
(b) Collector: Krylon, $Ba(OH)_2$

General Evaluation: Satisfactory, although collectors were saturated.

Response:

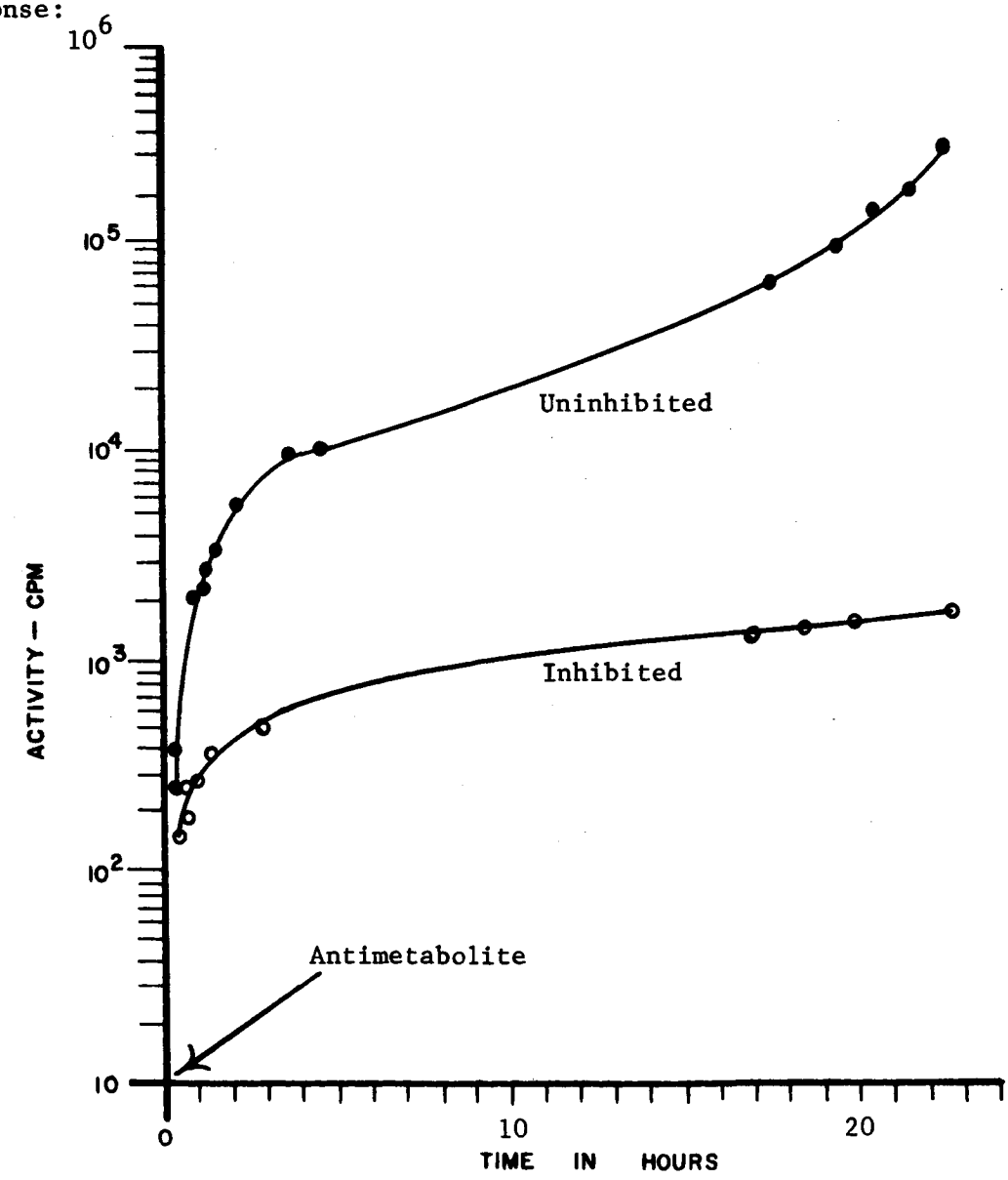


Figure II-9

FIELD TEST - GULLIVER III

Date: July 17, 1963

Weather: 33°C, sunny, slight wind

Location: Field - AMF, Alexandira, Va.

Orientation: Detector up

Ground Condition: Hard, dry, dusty

Soil Sample Collected: Very good

Medium: 3 ml, M8, 6.16 uc/ml

Antimetabolite: 0.5, Bard Parker

Radioactive Substrates:	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
Sodium formate	25.0	3.30	0.13	0.001
D-glucose-U.L.	4.7	0.66	0.14	0.003
DL-sodium lactate-1	5.0	2.20	0.44	0.005

Mechanical Function:

(a) Sequencing: No problems

(b) Projectiles: One projectile remained attached, released at port

(c) Thermostat: Not connected

Components:

(a) Detector: Geiger Müller tube

(b) Collector: AMF Tissuglas, $Ba(OH)_2$

General Evaluation: Satisfactory, although collectors became saturated.

Response:

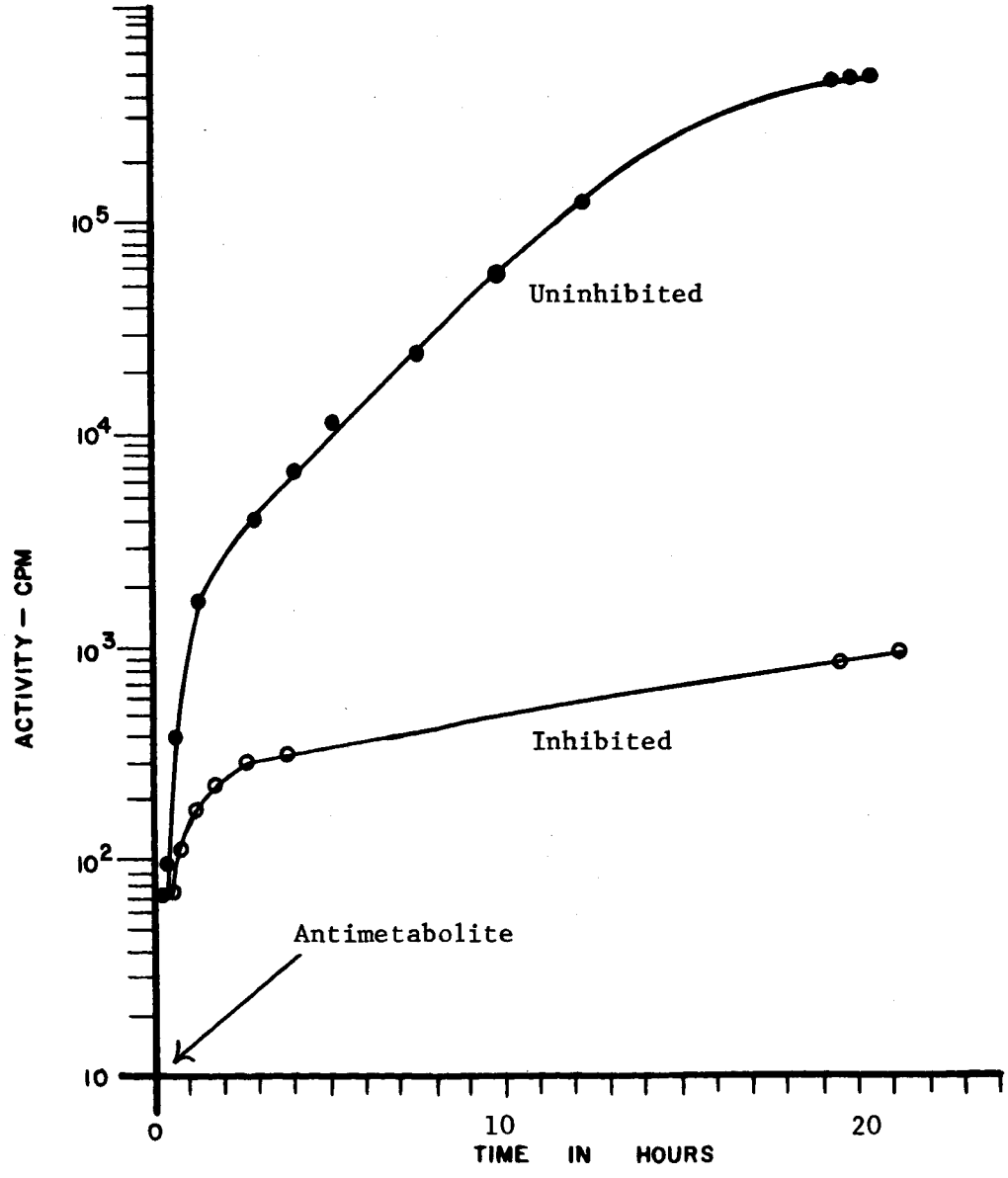
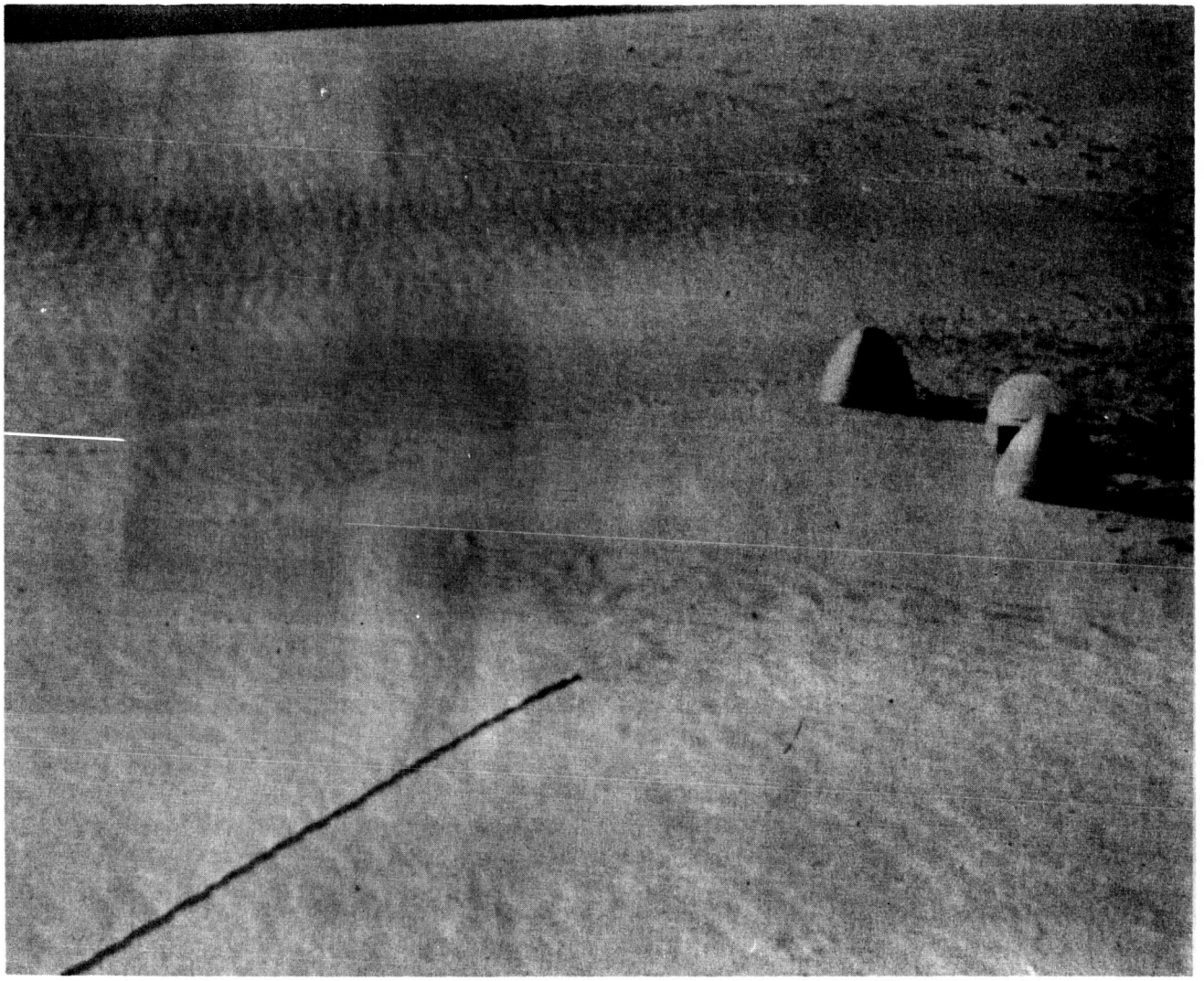


Figure II-10

EXTREME ENVIRONMENTAL FIELD TESTS



Death Valley, California

instruments functioned satisfactorily; the biological response was positive; and the antimetabolite, introduced after metabolism had been detected, was effective.

2. Sheep Mountain, White Mountain, California — Figure II-12, Frontispiece

This test was performed under very severe environmental conditions. The mountain site, located 12,000 feet above sea level, provided a barren expanse of jagged, flat, variously sized rocks, Frontispiece. Subfreezing weather ($-2^{\circ}C$), the low atmospheric pressure, and a subsequent blizzard compounded the adverse conditions present. Fifty minutes after commencement of the experiment, the blizzard necessitated complete termination of the test. The instruments, functioning most satisfactorily, would easily have withstood the elements, but the human factor, in fear of becoming stranded (a subsequent occurrence) decided that discontinuation would be the better part of valor under the circumstances.

With the exception of one projectile failing to free itself and one retrieval line snagging and breaking on the rocky surface, the mechanical operation was satisfactory. Biologically the test was eminently successful. In less than an hour a three-fold difference was present between the uninhibited unit, 1178 cpm, and the inhibited control unit, 520 cpm; demonstrating the ability of the instrument to perform and detect life in a short time under highly adverse conditions.

3. Death Valley, California — Figures II-13, II-10

The second California test was conducted on the Dunton Sand Dunes between Baker and Shoshone in the Death Valley area. The hot, dry environment and the scant amount of organic matter present in sand formed the basis for the site selection, Figure II-10. A very heavy soil sample

was obtained, partly due to the sandy nature of the soil, and partly to the added weight of one projectile (in each unit) failing to disengage itself. Responses were monitored for approximately 2.5 hours before darkness fell. In transporting the operating instruments to vehicles for continued monitoring during the night, the antimetabolite in the uninhibited unit was inadvertently triggered, causing inhibition in the test unit as well as in the previously inhibited control unit. Fortunately sufficient time had elapsed for a rapid, positive response to be registered.

4. Salton Sea, California Figures II-14, II-15, III-5

The third test was performed on the dry, alkaline, salt encrusted surface of the Salton Sea flats, Figure III-5. Although it had rained several days prior to the testing, the surface soil at the site was dry, resulting in the collection of a very heavy inocula. During the sampling, one collecting line broke; this unit was selected to be the uninhibited test, thus imposing severer testing conditions. The responses obtained indicated a successful test.

5. In Situ Determinations

In an effort to examine the possibility of detecting microbial life in situ on the planet's surface (without sample relocation), several tests were made in which the medium was placed directly on the ground's surface. Planchet collections were made. During the Death Valley and Salton Sea field trials, areas adjacent to the collecting site were selected for testing. One or two drops of the labeled M9 medium were placed on the soil or on rock. Planchets containing wet $Ba(OH)_2$ pads were inverted over the moistened area and the evolved $C^{14}O_2$ collected. The very high and rapid responses which resulted are presented in

Table II-1. To verify the true metabolic nature of the responses the soils were brought back to the laboratory for further testing. Following sterilization (to eliminate all microbial metabolic activity) planchet determinations were performed using the same M9-C^{14} field test medium. The non-metabolic activity produced by both soils was lower than the sterile medium control. (Additional study - Section III-B Soils). Since the preliminary efforts produced such promising results, further consideration will be given to possible instrument modification enabling in situ determinations.

Additional soil samples were collected by Resources Research personnel for laboratory testing in order to further substantiate the Gulliver responses, and also to determine the effects of two laboratory-developed media (Table II-3) on the soil microflora. Approximately 50 to 100 mg of soil were placed in planchets. One set received 0.25 ml of the field test medium, M9, developed specifically for soil microorganisms; a second set received 0.25 ml of the complex medium, M5. The responses, presented in Table II-1, demonstrate not only the importance of appropriate media development, but also greater sensitivity gained with the planchet method.

- - -

Measurements were obtained at the field sites and soil samples collected for analysis by Dr. Roy Cameron and Dr. Gerald Soffen of the Jet Propulsion Laboratory, Pasadena, California. Their results presented in Tables II-2A, B, C provided valuable information, particularly concerning the nature of the soils tested.

The entire field testing program proved to be highly successful. Although areas requiring improvement were indicated, rapid positive responses were obtained under adverse as well as normal conditions.

Figure II-11

FIELD TEST - GULLIVER III

Date: September 26, 1963

Weather: 20°C, sunny

Location: Orange, Virginia

Orientation: Detector up

Ground Condition: Dry, clay

Soil Sample Collected: Light

Medium: 3 ml, M8, 9.1 uc/ml

Antimetabolite: 0.5 ml Bard Parker

Radioactive Substrates:	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
(a) Sodium formate	25.0	6.5	0.26	0.002
(b) D-glucose U.L.	4.7	1.3	0.28	0.005
(c) DL-sodium lactate-1	5.0	1.3	0.26	0.002

Mechanical Function:

(a) Sequencing: No problems
(b) Projectiles: No problems
(c) Thermostat: Connected

Components:

(a) Detector: Geiger Müller tube
(b) Collector: AMF Tissuglas, LiOH

General Evaluation: Very satisfactory

Response:

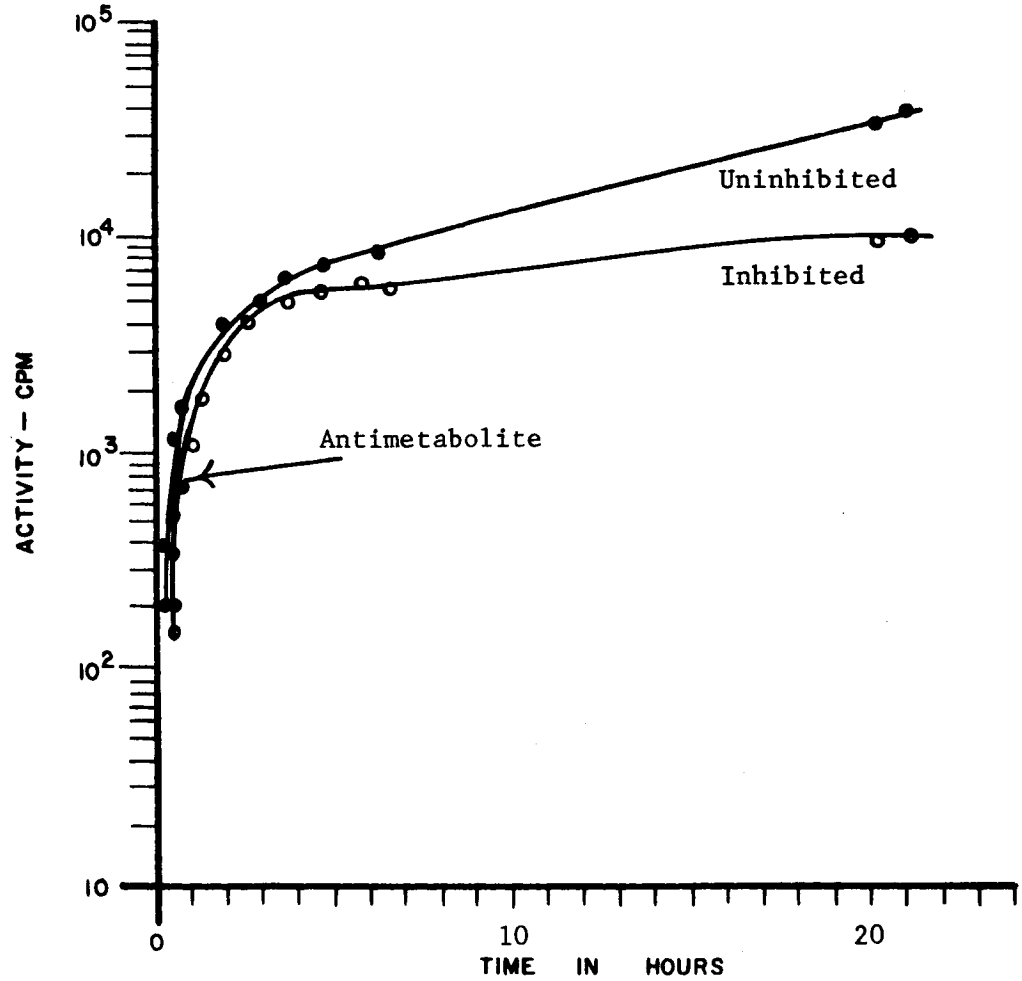


Figure II-12 FIELD TEST - GULLIVER III

Date: October 16, 1963

Weather: -2°C, windy

Location: Sheep Mountain, White Mountain, California

Orientation: Detector up

Soil Sample Collected: Light

Ground Condition: Mainly rock, little soil

Antimetabolite: 0.5 ml Bard Parker

Medium: 3 ml, M9, 9.66 uc/ml

Radioactive Substrates:	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
(a) Sodium formate	25.0	6.0	0.24	0.002
(b) D-glucose-U.L.	4.7	1.3	0.28	0.005
(c) DL-sodium lactate-1	5.0	1.3	0.26	0.002
(d) Glycine-1	4.4	1.0	0.22	0.002

Mechanical Function:
(a) Sequencing: No problems
(b) Projectiles: One projectile remained attached
(c) Thermostat: Functioning

Components:
(a) Detector: Geiger Müller tube
(b) Collector: AMF Tissuglas, LiOH

General Evaluation: Overall test was satisfactory. Collectors were saturated. An unexpected blizzard forced an early termination of the test.

Response:

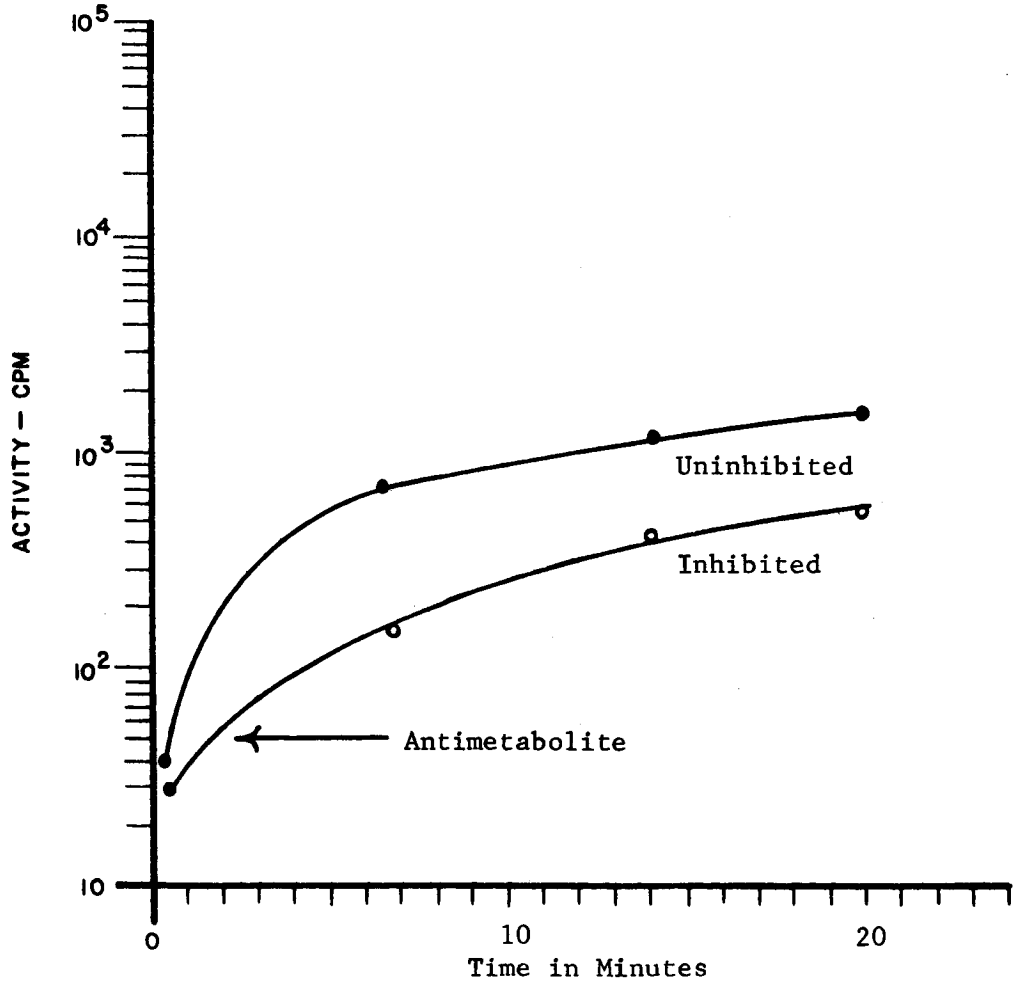


Figure II-13

FIELD TEST - GULLIVER III

Date: October 20, 1963 — Weather: 29°C, hot, sunny

Location: Death Valley, California — Orientation: Detector up

Ground Condition: Dry, sand dunes — Soil Sample Collected: Heavy

Medium: 3 ml, M9, 9.66 uc/ml — Antimetabolite: 0.5 ml Bard Parker

Radioactive Substrates:	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
(a) Sodium formate	25.0	6.0	0.24	0.002
(b) D-glucose-U.L.	4.7	1.3	0.28	0.005
(c) DL-sodium lactate-1	5.0	1.3	0.26	0.002
(d) Glycine-1	4.4	1.0	0.22	0.002

Mechanical Function:
- (a) Sequencing: No problems
- (b) Projectiles: Projectiles remained attached
- (c) Thermostat: Connected

Components:
- (a) Detector: Geiger Müller tube
- (b) Collector: AMF Tissuglas, LiOH

General Evaluation: Overall test was satisfactory. Projectiles failed to carry free in both units, but actually increased the amount of sample by adding weight to the lines. Antimetabolite was inadvertently introduced into the test unit sometime between 2.5 hours and 3.5 hours.

Response:

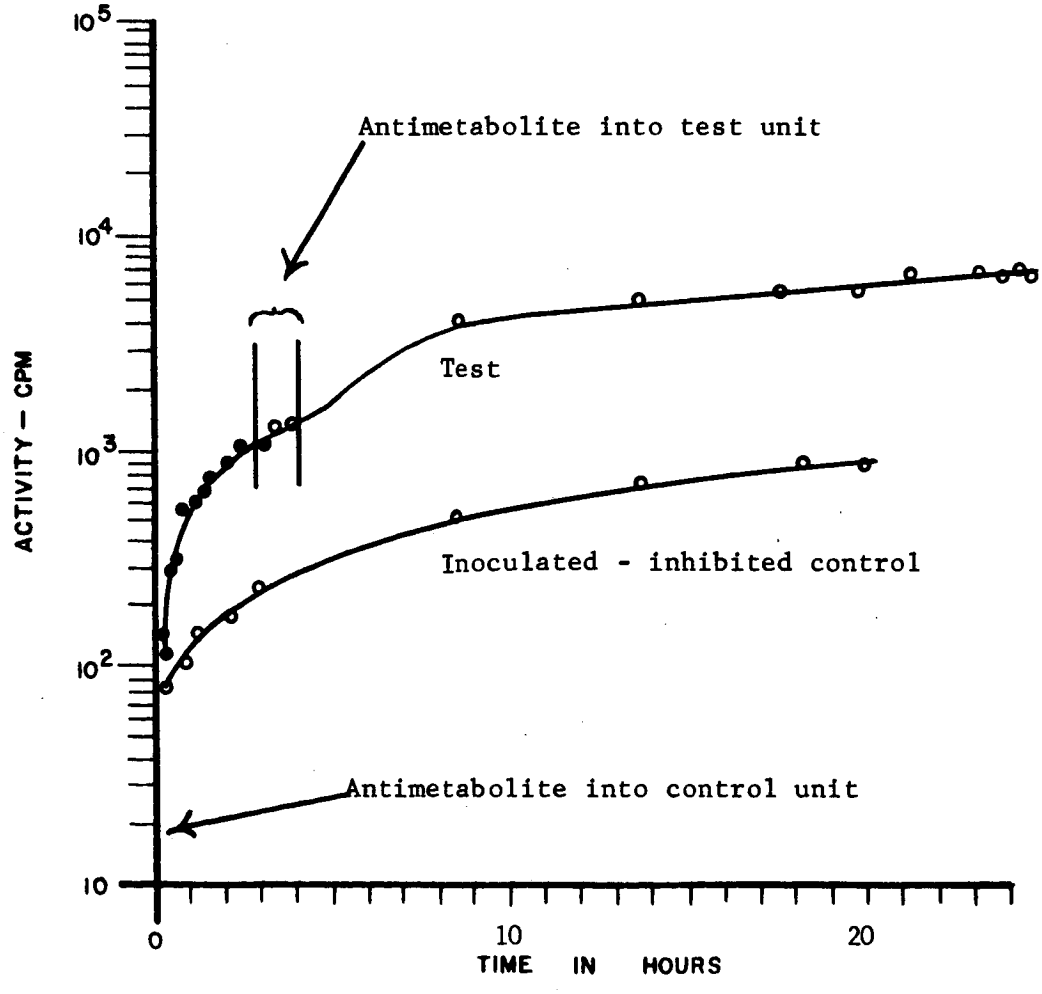


Figure II-14

FIELD TEST - GULLIVER III

Date: October 25, 1963

Weather: 32°C, recent rain

Location: Salton Sea, California

Orientation: Detector up

Ground Condition: Hard, granular

Soil Sample Collected: Light

Medium: 3 ml, M9, 9.66 uc/ml

Antimetabolite: 0.5 ml Bard Parker

Radioactive Substrates:	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
(a) Sodium formate	25.0	6.0	0.24	0.002
(b) D-glucose-U.L.	4.7	1.3	0.28	0.005
(c) DL-sodium lactate-1	5.0	1.3	0.26	0.002
(d) Glycine-1	4.4	1.0	0.22	0.002

Mechanical Function:

(a) Sequencing: No problems

(b) Projectiles: One retrieval line broke

(c) Thermostat: Connected

Components:

(a) Detector: Geiger Müller tube

(b) Collector: AMF Tissuglas, LiOH

General Evaluation: Overall test was satisfactory. One retrieval line broke. Uninhibited test conducted in chamber with single line.

Response:

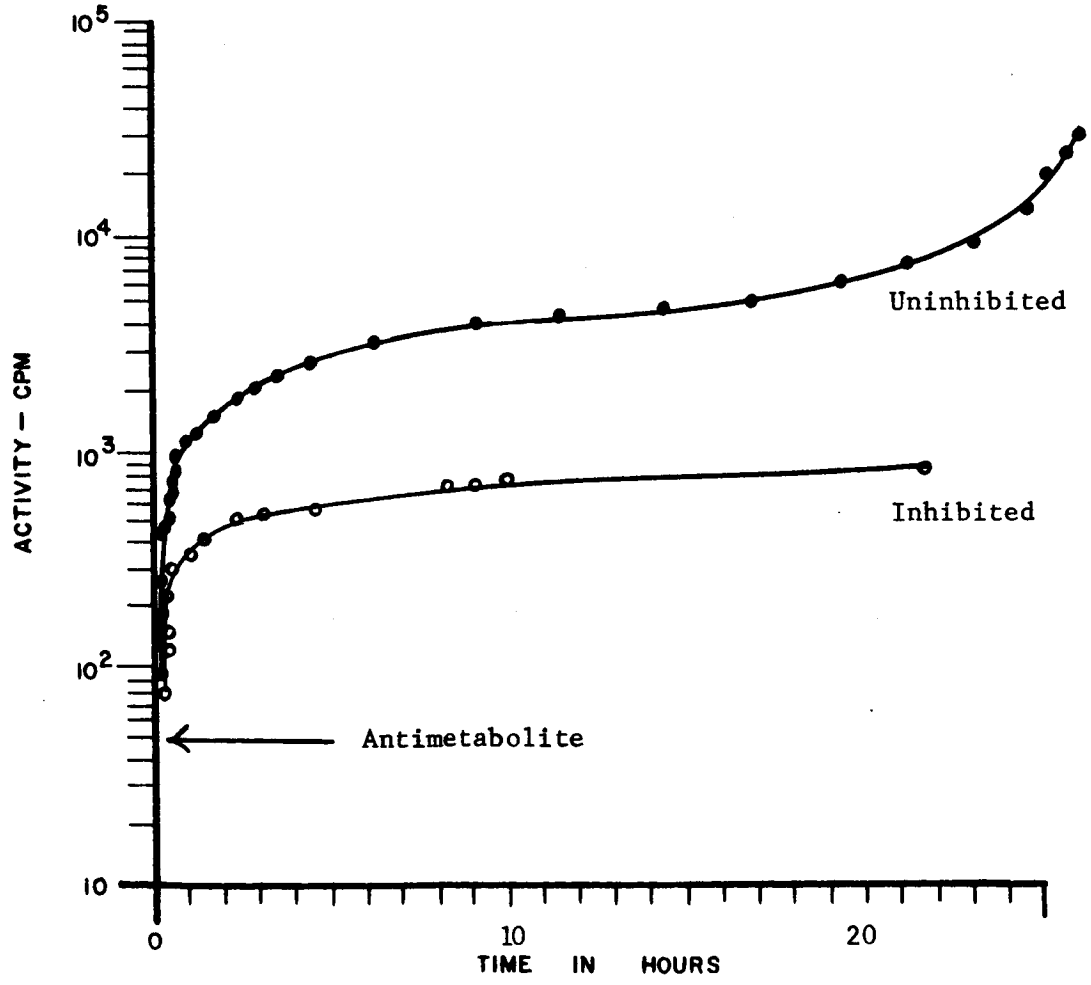


Figure II-15

SALTON SEA, CALIFORNIA FIELD TEST

RESPONSE - FIRST FIVE HOURS

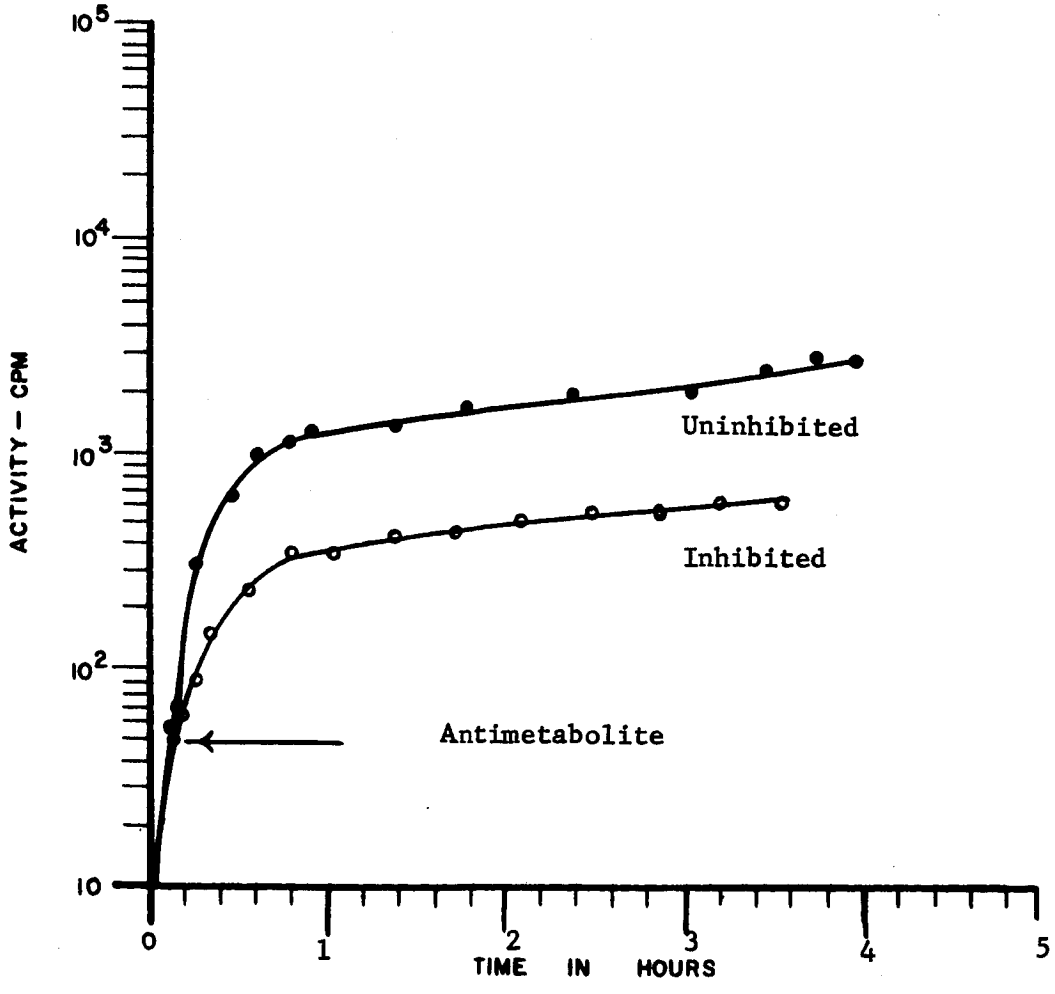


Table II-1

RESPONSE FROM CALIFORNIA FIELD TEST SOILS

PLANCHET DETERMINATION

Soil		Successive Incubation Periods* (Hours)	Net Radioactivity - CPM Medium (0.25 ml) M5	M9
Sheep Mt.				
Soil	50-100 mg	1.25	4,389	50,481
		4.00	3,240	27,055
Pebbles	50-100 mg	1.25	4,229	53,866
		4.00	3,177	30,413
Death Valley				
Soil depth	0.25 in.	0.75	12	441
75 mg		1.50	22	605
Soil depth	3.00 in.	0.75	34	370
75 mg		1.50	42	555
Salton Sea				
50-100 mg		1.00	2,156	22,439
		2.00	1,712	22,947

In Situ Determination

Death Valley			
Direct soil	1.00**	(1 drop medium)	40,000
Salton Sea			
Direct rock	0.50**	(1 drop medium)	1,980
Direct soil	1.00**	(2 drops medium)	40,000

* Incubation periods include 15 minute $C^{14}O_2$ collection period.

** Continuous $C^{14}O_2$ collection-incubation period.

Table II-2A

REPORT* ON RESULTS OF SOIL TEST SITES FOR "GULLIVER" III LIFE DETECTION

Test Site 1:

Near Sheep Mountain, White Mountain Range, Inyo National Forest California, October 16, 1963; soil #43. Elevation of site: 11,750 ft. Air temp.: 29°F; soil surface temp. (shade, no sun): 29°F; air relative humidity, 3 ft. above soil surface (just preceding snow flurries): 78% RH. Wind direction, variable, generally from the northwest; gusts up to 20-25 mph. Soil moisture (including pebbles), 7.7%. Mechanical analysis: 84.9% material above 2 mm (primarily dolomite with adherent lichens and/or associated moss and other organic matter); 43.4% sand (2 mm - 2 mu), 37.5% silt (20 mu - 2 mu), 19.1% clay (2 mu or less). Soil textural class: angular coarsely gravelly loam. Bacteriological fallout plate counts on trypticase soy agar with up to ten days incubation at 25°C after return to lab: one-minute average, no counts taken; five-minute average, 32 per square inch; ten-minute average, 274 per square inch (snowfall occurred during time of plate exposure). Aerobes (incubation at 25°C, January 7-13 with air dry soil, trypticase soy agar plates): sample fraction less than 2mm, $1,460 \times 10^3$ per gm of soil; sample fraction less than 2mm (ground-up sample) 105×10^3 per gm of soil.

* Roy E. Cameron, Gerald A. Soffen
Jet Propulsion Laboratory, Pasadena, California

Table II-2B

REPORT ON RESULTS OF SOIL TEST SITES FOR "GULLIVER" III LIFE DETECTOR

Test Site 2:

Dunton Sand Dunes east of Highway 127, 4 miles southwest of Shoshone, between Shoshone and Tecopa, California, October 20, 1963; soil #44. Elevation 800 ft. Air temp., 84°F; soil surface temp. (sun), 89°F; soil surface temp. (shade), 83°F; air relative humidity 3 ft. above soil surface temperature, 47% RH. Wind direction: east by southeast; wind velocity 7 mph. Soil moisture values: surface 1/4", 0.9%; 2" level, 2.2%; 3" level, 0.8%. Mechanical analysis: % material above 2 mm, 0.0%; 89.6% sand, 1.7% silt, 8.7% clay. Soil textural class: dune sand. Bacteriological fallout plate counts: one-minute average, 0.2 per square inch; 5-minute average, 0.9 per square inch; 10-minute average, 1.0 per square inch. Aerobes (same conditions as stated above): sample fraction less than 2 mm, 51×10^2 per gm soil; 3" level, 41×10^2 per gm of soil.

Table II-2C

REPORT ON RESULTS OF SOIL TEST SITES FOR "GULLIVER"III LIFE DETECTOR

Test Site 3:

West of Salton Sea, approximately 8 miles south of Salton City and approximately 6 miles west of Salton Sea gauging station, October 25, 1963; soil #50. Elevation - 50 ft.; air temp. at 3' above the soil surface 87-90°F; soil surface temp. (sun), 112° F; soil surface temp. (shade), 100°F, air relative humidity at 3' above the soil surface, 33% RH. No noticeable wind. Soil moisture values: surface 1/4", 2.0%; 1/4" - 2", 5.0%. Mechanical analysis: 30.7% material above 2mm (sandstone, silt stone, and clay stone), 44.4% sand, 17.0% silt, 38.6% clay. Soil textural class: clay loam. Bacteriological plate counts: 1-minute average, 0.3 per square inch; 5-minute average, 1.1 per square inch; 10-minute average, 1.6 per square inch. Aerobes: sample fraction less than 2 mm, 361×10^3 per gm of soil; sample fraction more than 2 mm (ground-up sample), 165×10^3 gm soil.

Table II-3

COMPOSITION OF FIELD TEST MEDIA

Medium M9

C^{14} substrates	* ---
K_2HPO_4	1.0 g
KNO_3	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
Soil extract per liter	100.0 ml

Medium M8

C^{14} substrates	** ---
K_2HPO_4	1.0 g
KNO_3	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
per liter	

Medium M5

C^{14} substrates	* ---
K_2HPO_4	1.0 g
KNO_3	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
$FeCl_3$	0.01
Na_2SO_3	0.2
Malt extract	3.0
Beef extract	3.0
Yeast extract	13.0
Ascorbic acid	0.2
L-cystine	0.7
Bacto casamino acid	4.0
Proteose peptone #3	20.0
Soil extract per liter	250.0 ml

* C^{14} substrates M5, M9

	Sp. Act. (mc/mM)	uc/ml	mM/l	% (w/v)
Sodium formate	25.0	6.0	0.24	0.002
D-glucose-U.L.	4.7	1.3	0.28	0.005
DL-sodium lactate-1	5.0	1.3	0.26	0.002
Glycine-1	4.4	1.0	0.22	0.002
Total		9.6	1.00	0.011

** C^{14} substrates M8

Sodium formate	25.0	6.5	0.26	0.002
D-glucose-U.L.	4.7	1.3	0.28	0.005
DL-sodium lactate-1	5.0	1.3	0.26	0.002
Total		9.1	0.80	0.009

III. BIOLOGICAL INVESTIGATION

During the past year, progress has been made toward optimization and refinement of the Gulliver experiment. The basal medium has been improved by numerous studies involving the labeled and unlabeled constituents. Growth additives, concentration levels and ratios, increased specific activities and new labeled substrates have all been under investigation. The development and performance of the various media were dependent upon, and evaluated from, the responses elicited by the wide range of pure cultures and soils in the test collection. The ability of Gulliver to detect metabolism under strict anaerobic conditions and the feasibility of adapting the radioisotopic method to detect photosynthetic life was demonstrated.

A. MEDIUM DEVELOPMENT

The principal biological objective of the program is the development of a labeled medium which will support the growth of the greatest possible number of microbial species. The constituents of this medium must not only be nutritionally beneficial but must also be metabolized and incorporated into an evolved gas which contains tagged atoms originally present in the medium. The selection, concentration, and interrelation of various compounds have been studied. An evaluation was made of responses obtained from a wide variety of microorganisms cultured in the resultant medium. Consequently, a series of media has been developed.

Three types of media are currently under investigation: (1) M8 - a simple salts medium containing no organic constituents except for the labeled compounds, (2) M5 - a complex medium incorporating the inorganic salts of M8 as well as various organic compounds, and

(3) M9 - a medial version of the first two. The complex medium was selected to support the growth of a wide range of microorganisms; the simple medium was tailored more specifically for soil organisms and for maximizing the utilization of the labeled compounds; the 'intermediate' medium was designed to lessen potential labeled substrate-organic competition, reduce possible inhibitory concentrations or organic materials, and to minimize the dilution of the labeled compounds. Thus, the M9 is the currently preferred candidate to support the maximum possible number of species.

The program commenced with the use of the basal medium, M8-formate-glucose-C^{14}, the product of the various medium developmental stages, M1 - M7 (First and Second Annual Progress Reports). Successive studies during the current year resulted in (1) M8 containing formate-glucose-lactate-C^{14} (2) M9 with formate-glucose-lactate-C^{14} and (3) M9 with formate-glucose-lactate-glycine-C^{14} substrates. The complex M5 medium was used comparatively and concurrently with the others. Table III-1 lists the constituents of the various media.

It is apparent from the foregoing paragraph that emphasis has been placed upon relatively simple basal media throughout most of the year. The absolute responses have generally been higher and more rapid, particularly from soil inocula for which they were primarily developed. However, despite attainment of greater initial sensitivity, further increases of activity were not observed after several hours of incubation in laboratory experiments. Comparisons with sterile control levels indicated that metabolism was initially present, but was not followed by growth of the organisms. This lack of growth may be the result of insufficient nutrients compounded by inadequate amounts of available CO_2 or O_2, as well as $C^{14}O_2$ collector saturation. The limited growth curve rarely occurred with the

Table III-1

COMPOSITION OF BASAL MEDIA

C^{14} Substrates Not Included

	Constituents	Concentration (g/l)
Medium M9	K_2HPO_4	1.0
	KNO_3	0.5
	$MgSO_4 \cdot 7H_2O$	0.2
	NaCl	0.1
	Soil extract	100.0 ml/l
Medium M8	K_2HPO_4	1.0
	KNO_3	0.5
	$MgSO_4 \cdot 7H_2O$	0.2
	NaCl	0.1
Medium M5	K_2HPO_4	1.0
	KNO_3	0.5
	$MgSO_4 \cdot 7H_2O$	0.2
	NaCl	0.1
	$FeCl_3$	0.01
	Na_2SO_3	0.2
	Malt extract	3.0
	Beef extract	3.0
	Yeast extract	13.0
	Ascorbic acid	0.2
	L-cystine	0.7
	Bacto casamino acid	4.0
	Proteose peptone #3	20.0
	Soil extract	250.0 ml/l

actual Gulliver instrument in field trials, indicating that differences in test material or equipment and chamber design may be responsible.

Since the simple salts medium containing labeled organic substrates appeared to be a good foundation, the medium development efforts were directed toward gradual nutritional supplementation of the M8. It was hoped that the medium could be sufficiently enriched to promote growth of those organisms requiring complex substrates without building it to the point where it would inhibit other organisms, principally, the autotrophs. Experimentation was divided into two parallel but interrelated phases - the study of labeled substrates and the study of non-radioactive substrates. Although the two are discussed separately, they are nevertheless closely dependent upon one another and evaluation must be, and has been, based upon the combined results.

1. Basal Media Substrates - Unlabeled

Supplementation of the simple salts medium, M8, with soil extract, Panmede, and sodium succinate was investigated. Planchet as well as automatic recorder determinations were performed on pure cultures and soils. The use of soil extract in the basal medium had been tried previously. The basal media M5 - M7 contained 250 ml/liter of soil extract; however, they also contained a high percentage of organic compounds. A study performed on soils, cultured in a labeled soil extract medium (Second Annual Progress Report) indicated that some advantage might be obtained by adding unlabeled extract in a lower concentration, 100 ml/l, to the M8. Panmede, a medium supplement containing liver digest, vitamins, amino acids, and trace elements (supplied by Paines and Byrne , Ltd.) was simultaneously investigated as a single additive and in combination with soil extract. The results, presented in Table III-2

Table III-2

MEDIA COMPARISON - M8 CONTAINING SOIL EXTRACT, PANMEDE, AS ADDITIONAL GROWTH FACTORS

PLANCHET DETERMINATION

Inoculum	Successive Incubation Periods* (Hours)	Net Radioactivity - CPM Medium**			
		M8	M8 Panmede	M8 Soil Extract	M8 Soil Extract Panmede
Apple Valley	1	3,086	2,815	3,233	2,792
100 mg	2	2,899	2,840	3,220	2,900
	4	2,730	3,234	2,867	2,642
Metuchen Soil	1	52,742	58,344	66,698	60,650
100 mg	2	17,576	33,111	34,910	42,178
	4	4,948	3,063	4,370	3,478
Florida Saline Soil	1	62	36	21	37
100 mg	2	59	0	80	70
	4	68	219	172	617
E. coli	1	9,279	9,675	9,133	8,982
2.7×10^6	2	17,871	25,454	21,412	31,287
cells/test	4	51,166	50,504	50,046	50,400
B. subtilis	1	8,655	12,338	14,308	12,382
var globigii	2	21,173	41,967	39,730	44,896
1.4×10^6 cells/test	4	73,691	24,158	50,235	30,350
Media Controls	1	43	125	74	118
	2	60	176	56	146
	4	49	146	44	141

* Each incubation was followed by a 15 minute $C^{14}O_2$ collection period.

** C^{14} substrates	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL-sodium lactate-1	5.0	0.26	1.3	0.002

were marginal. Since the Panmede caused slightly higher control levels and did not produce compensatory responses, it was not considered further. The soil extract, however, was considered to be sufficiently beneficial for inclusion in the medium. The new medium resulting from the addition of 100 ml/l of soil extract to the labeled M8 medium was designated M9.

An indirect approach toward obtaining greater sensitivity was investigated by supplementing the medium with a compound which would increase the utilization of the labeled nutrients, and consequently, increase the $C^{14}O_2$ production. G. V. Levin, in Reducing Secondary Effluent Phosphorus Concentration, Progress Report #1, District of Columbia and Public Health Service, April, 1963, showed that ortho-phosphate uptake by sewage microorganisms fed glucose was increased by the substitution of sodium succinate for some of the glucose to provide the same total moles of substrate, but in a 3/1 succinate/glucose molar ratio. However, when glucose was totally omitted, considerably less uptake occurred. Hence, it seemed that the succinate might increase utilization of the glucose. Since glucose-C^{14} is a prime substrate of the basal media it was possible that its utilization here also could be increased by the presence of sodium succinate. Since labeled succinate was not immediately available, unlabeled succinate was added in the suggested ratio to the M8 medium and determinations performed on the automatic recording unit. The general response (Table III-3) did not indicate any increase of detectable $C^{14}O_2$. Because it is possible that the sodium succinate competed with the labeled glucose, experimentation is continuing with labeled succinate.

All of the media developed and tested, M1 - M9, have contained labeled glucose as an important energy source as well as a prime source of carbon for synthesis. The limited growth curves observed with the simple media indicated the possibility of an inadequate supply of glucose, and the necessity to evaluate increased concentrations of this important constituent. The medium in use, M9, contained a total concentration of 0.005% w/v of uniformly labeled glucose. Determinations were performed (details are given below) in which successively greater concentrations of unlabeled glucose were added to the existing 0.005% glucose-C^{14} until 1.005% w/v was present.

Method ----- Automated monitoring and recording system

Medium ----- M9, 0.5 ml

C^{14} Substrates -----	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL-sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.4	0.22	1.0	0.002

Inocula ----- Apple Valley soil 100 mg
Rocky Mountain soil 75 mg
E. coli - tryptone - glucose broth inoculum 2×10^6 cells
S. cerevisiae 9×10^3 cells

Total incubation and collection period ----- Continuous for 18 hours

Concentration of glucose ----- 0.005, 0.055, 0.505, 1.005% w/v (0.005% was radioactive in each medium)

Results ----- All concentrations, with one exception, produced similar responses. The medium containing the lowest concentration, 0.005% w/v, produced responses comparable to the highest concentration, 1.005% w/v. Table III-3 presents the data from two typical responses. The only exception, and an interesting one, was obtained from *E. coli*, Figure III-1. This inoculum, prepared in tryptone-glucose broth, produced the only definitely exponential growth response. In order to evaluate this response

Table III-3

MEDIA COMPARISON - M8, M9 CONTAINING SODIUM SUCCINATE, UNLABELED GLUCOSE, AS ADDITIONAL GROWTH FACTORS
RECORDER DETERMINATION

ADDITION OF SODIUM SUCCINATE - MEDIUM M8* (0.5 ml)

Inoculum	Continuous $C^{14}O_2$ Collection Incubation Periods (Hours)	Radioactivity - CPM Medium M8* (0.5 ml) M8	M8 Sodium Succinate
E. coli 2.1×10^6 cells/test	1	380	350
	3	1,120	960
	5	2,200	1,800
	17	19,600	13,000

* C^{14} Substrates -----	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	5.0	1.00	5.0	0.007
D-glucose-U.L.	3.0	0.33	1.0	0.006

INCREASED UNLABELED GLUCOSE CONCENTRATION

Inoculum	Continuous $C^{14}O_2$ Collection Incubation Periods (Hours)	Radioactivity - CPM Medium M9** (0.5 ml) (includes 0.005% glucose-C^{14}) M9 0.005% glucose-C^{14}	M9 0.055% glucose	M9 0.505% glucose	M9 1.005% glucose
Rocky Mountain Soil 75 mg	1	12,000	15,000	12,000	12,000
	3	28,000	33,000	27,000	25,000
	5	36,000	40,000	33,000	28,000
	12	43,000	43,000	43,000	41,000
S. cerevisiae 9.8×10^3 cells/test	1	630	640	570	310
	3	830	880	850	510
	5	1,800	1,350	900	1,050
	18	3,600	2,200	1,600	1,800

** C^{14} Substrates -----	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL-sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.0	0.22	1.0	0.002

further, and also to determine if an increased concentration of labeled glucose produced responses similar to those from an identically increased concentration of cold glucose (containing, in addition, 0.005% of the basic glucose-C^{14}), a second set of determinations were performed in the following manner.

Method ----- Automated monitoring and recording system

Medium ----- M9, 0.5 ml

C^{14} Substrates -----	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	4.7	(varied as below)		
DL-sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.4	0.22	1.0	0.002

Inocula ----- Apple Valley soil 100 mg
Rocky Mountain soil 50 mg
E. coli - Nutrient Broth inoculum 1×10^6 cells
S. cerevisiae 1×10^4 cells

Total incubation and collection time ----- Continuous for 18 hours

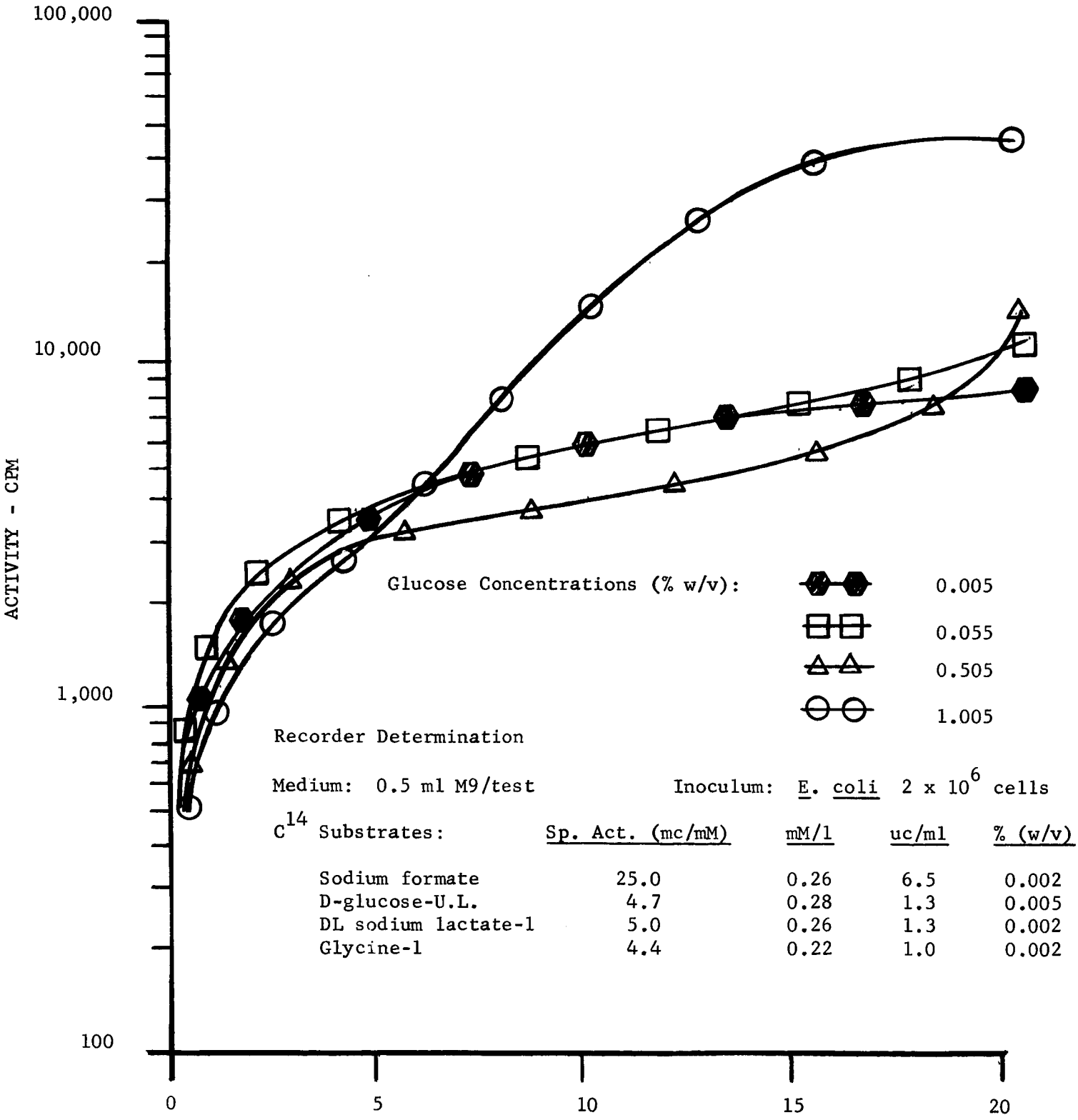
Concentration of glucose ----- Medium (1) 0.005 labeled
Medium (2) 0.205 labeled
Medium (3) 0.200 unlabeled, 0.005 labeled

Results ----- Both the unlabeled and labeled glucose produced similar responses; only metabolism was detected from all inocula, including the *E. coli*, which had been cultured prior to use in Difco Nutrient Broth rather than the tryptone-glucose broth.

The general observation from both determinations was that the addition of either labeled or cold glucose up to 1.005% was not, in itself, sufficient to produce the desired growth response, thus indicating that some other nutritional factor might be lacking. Further indication of this was given by the responses of the tryptone-glucose cultured and the Nutrient Broth cultured inocula of *E. coli*. The possibility that some of the constituents of the tryptone-glucose broth influenced the

Figure III-1

RESPONSE OF ESCHERICHIA COLI TO CONCENTRATIONS OF GLUCOSE IN M9 MEDIUM



growth of the E. coli in the M9 led, in part, to the next series of investigations.

- - -

A number of compounds were tested as growth supplements to the M9 medium. Among these were two constituents of the tryptone-glucose broth, tryptone and beef extract; peptone and yeast extract, which are present in the M5 medium (as is the beef extract); sodium pyruvate; and additional nitrogen sources - asparagine, ammonium chloride and potassium nitrate. Also included was M5 medium, diluted with water to one fifth concentration, and designated 1/5 M5. The experimental details were as follows:

Method ----- Planchet; automated monitoring and recording system

C^{14} Media ----- 0.5 ml

(1) M9
(2) M5
(3) 1/5 M5
(4) Tryptone-glucose broth
(5) M9 plus 1 g beef extract, 1 g tryptone/1
(6) M9 plus 2 g beef extract, 2 g tryptone/1
(7) M9 plus 2 g beef extract, 4 g tryptone/1
(8) M9 plus 3 g beef extract/1
(9) M9 plus 5 g peptone/1
(10) M9 plus 5 g peptone, 3 g beef extract/1
(11) M9 plus 6.5 g yeast extract/1
(12) M9 plus 6.5 g yeast extract, 0.1 g sodium pyruvate/1
(13) 1/5 M5 plus 0.1 g sodium pyruvate/1
(14) M9 plus 0.4 g/1 asparagine (total N source 0.5 g/1)
(15) M9 plus 0.3 g/1 ammonium chloride (total N source 0.5 g)
(16) M9 plus 0.5 g/1 potassium nitrate (total N source 0.5 g/1)

C^{14} Substrates -----	Sp. Act. (mc/mM)	mM/1	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL-sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.4	0.22	1.0	0.002

Inocula ----- Death Valley soil 100 mg
Iron-rich soil 100 mg
E. coli
B. subtilis var globigii

Total incubation and collection time ----- Recorder determinations - continuous for an average of 18 hours
Planchet determination-time intervals varied.

Results ----- Tables III-4, III-5. Generally, there were no dramatic responses from any of the media. Closer evaluation revealed areas where slight advantage may have been gained, or might be obtained with varied concentrations or dilutions. E. coli produced its best responses from the 1/5 M5 and the M9 media containing beef extract or peptone or yeast extract. The Death Valley soil specifically used for its low total cell numbers responded poorly in all media, indicating after 19 and 21 hours of incubation the use of 1/5 M5 and M9 plus yeast extract. Conversely, the iron-rich soil was selected for its high microbial content. This soil from Orange, Va., presented the most interesting responses. In M9 it produced the highest response within the first hour and then gradually decreased; with M5 a low initial response increased to a relatively high one during the fifth hour; the 1/5 M5 resulted in a relatively high initial response followed by a substantially higher one in five hours. When the E. coli and soil responses were correlated, it appeared that the diluted M5, and the M9 containing either beef extract, peptone, or yeast extract (all of which are also present in the M5 and diluted to lower concentrations in the 1/5 M5) were beginning to produce the desired responses. This indicated again, that the M5 was too rich organically and the M9 too limiting. The medium desired may be a compromise between the 1/5 M5 and the M9.

Presently under investigation are organic compounds which can supplant carbon dioxide, an essential and possibly limiting nutrient in the closed incubation chambers of the automated laboratory culturing device. The intense effort to determine the cause or causes of the limited growth curve will continue throughout the forthcoming year.

Table III-4

MEDIA COMPARISON - M9* CONTAINING BEEF EXTRACT, PEPTONE, TRYPTONE, NITROGEN SOURCES, AS ADDITIONAL GROWTH FACTORS

RECORDER DETERMINATION

Inoculum	$C^{14}O_2$ Continuous Collection Incubation Periods (Hours)	Net Radioactivity - CPM Medium (0.5 ml) M9	M9 asparagine	M9 ammonium chloride	M9 potassium nitrate
Iron-rich Soil (Orange, Va.) 50 mg	1	10,320	11,800	12,000	11,640
	3	21,800	26,800	18,900	25,400
	5	28,800	35,800	23,800	32,200
	19	47,800	47,700	41,800	47,000
Death Valley Soil 100 mg	1	800	1,000	1,000	450
	3	1,300	1,560	1,100	780
	5	1,500	2,000	1,530	1,100
	17	2,640	3,160	2,000	1,410

		M9	M9 beef extract	M9 peptone	M9 beef extract peptone
Death Valley Soil 100 mg	1	750	470	700	700
	3	1,000	700	1,000	800
	5	1,500	930	1,600	900
	19	1,740	1,200	4,000	2,700
E. coli 8.6×10^5	1	2,520	2,560	2,500	2,340
	3	3,420	6,260	5,660	7,140
	5	4,000	13,660	8,600	12,700
	17	5,800	47,700	33,000	32,600

		Tryptone-glucose Broth beef extract 3 g/l tryptone 5 g/l	M9	M9 beef extract 1 g/l tryptone 1 g/l	M9 beef extract 2 g/l tryptone 2 g/l	M9 beef extract 2 g/l tryptone 4 g/l
Death Valley Soil 100 mg	1	700	800	750	750	700
	3	1,000	1,000	900	900	1,050
	5	1,200	1,100	1,050	1,200	1,100
	17	1,275	1,350	1,200	1,300	1,800

* C^{14} Substrates -----

	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL-sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.4	0.22	1.0	0.002

Table III-5

MEDIA COMPARISON - M5, 1/5 M5, M9 CONTAINING YEAST EXTRACT, SODIUM PYRUVATE, AS ADDITIONAL GROWTH FACTORS

PLANCHET DETERMINATION

Inoculum	Successive Incubation Periods* (Hours)	Net Radioactivity - CPM Medium** (0.5 ml)					
		M5	M9	1/5 M5	M9 yeast extract	1/5 M5 pyruvate	M9 yeast extract pyruvate
Iron-rich Soil (Orange, Va.) 100 mg	1	14,765	49,332	32,782	46,106	25,799	39,261
	3	13,018	36,448	26,574	41,518	23,988	36,165
	5	40,505	35,919	56,832	42,729	25,042	33,839
	21	2,797	813	2,217	2,034		
Death Valley Soil 100 mg	1	100	267	247	237	312	560
	3	94	263	234	154	295	556
	5	77	218	279	168	327	524
	21	8,986	206	37,986	35,300		
Escherichia coli 1.4×10^6 cells/test	1	8,639	15,798	16,650	21,347	21,183	47,950
	3	36,592	34,699	75,705	76,773	10,937	16,918
	5	7,215	59,885	24,365	15,596	34,687	46,439
	21	3,039	69	322	371		
B. subtilis var globigii	1	67	30	114	141		
	3	15	7	58	1,971		
	5	21	52	158	26,726		
	21	22,831	151	9,629	3,505		

* Each incubation period was followed by a 15 minute $C^{14}O_2$ collection period.

** C^{14} Substrates -----

	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	3.2	0.43	1.3	0.007
DL-sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.4	0.22	1.0	0.002

2. Basal Media Substrates - Labeled

a. C^{14} Labeled Substrate Concentration
Effects of Increased Specific Activity

Attainment of maximum sensitivity has been a prime objective throughout the program. The concentration and radioactive levels of C^{14} substrates in the medium are directly related to the problem. Not only must sufficient amounts of tagged nutrients be available to the organisms, but they must also be at the most advantageous radioactive levels and ratios. The compounds primarily involved, formate-C^{14} and glucose-C^{14}, constitute the standard radioactive substrates of the medium. Previous determinations (Second Annual Progress Report) on formate of 5 mc/mM specific activity and glucose of 3 mc/mM specific activity indicated that a molar ratio of 3 formate/1 glucose and a radioactive ratio of 5 formate/1 glucose were the most advantageous. The substrates were incorporated in the basal medium at the specified levels.

The study, continued during the current year, investigated the substrates at higher specific activity levels. Formate was obtained at 25 and 5 mc/mM and glucose at 12 and 4.7 mc/mM. Various combinations were compared on a molar and radioactive basis. Since the formate and glucose compose a standard pair, the compounds were tested as a set and not individually. However, in order to validly determine the effect of the substrate with the increased specific activity, the other compound was maintained at a constant level. A third compound, DL-sodium lactate-1-C^{14}, was subsequently added to the regular C^{14} substrates. It was incorporated at a constant level throughout the remaining determinations. The experimental details are summarized below.

Method ----- Automated monitoring and recording system

Medium ----- M8 or M9, 0.5 ml

C^{14} Substrates -----

1. Formate Determinations - Table III-6
2. Glucose Determinations - Table III-7

Inocula ----- Soil (100 mg/test) Organisms (0.1-0.2 ml/test)

Field soil	E. coli
Garden soil	S. cerevisiae
Apple Valley soil	R. rubrum
Rocky Mountain soil	S. marcescens

Total incubation and collection period ----- continuous for 18 hours

Results ----- Figure III-2. Formate of the higher specific activity, 25 mc/mM, produced better responses at both molar concentrations than that of the lower 5 mc/mM activity. Although the 1.0 mM/1 (at 25 mc/mM) produced the best results, the 0.20 mM/1 concentration (at 25 mc/mM) was selected. The lower molar concentration would not increase the total molar concentration of the medium constituents (large concentrations might become inhibitory) and the resultant lower radioactive level (5 uc/ml) would not contribute additional undesirable non-metabolic activity.

The glucose determinations which followed, incorporated, at constant levels, the high activity formate as well as the newly added lactate-C^{14}. The results of the various combinations were surprisingly similar. It appeared that increasing the specific activity of glucose did not offer any advantage when used with the double or triple-labeled medium.

- - -

The addition of a new basic substrate, sodium lactate-C^{14}, and the considerable increase in the specific activity of formate used as well as a slight increase of 3 to 4.7 mc/mM of newly obtained glucose changed the individual and total molar levels of the tagged constituents, making it necessary to determine the effects of the alteration.

Table III-6

COMBINATIONS TESTED FOR EFFECTS OF INCREASED SPECIFIC ACTIVITY OF FORMATE-C^{14}

Combination	Specific Activity mc/mM	mM/l	Radioactivity
formate	25	1.00	25
glucose	3	0.33	1
		1.33	26
formate	25	0.20	5
glucose	3	0.33	1
		0.53	6
formate	5	1.00	5
glucose	3	0.33	1
		1.33	6

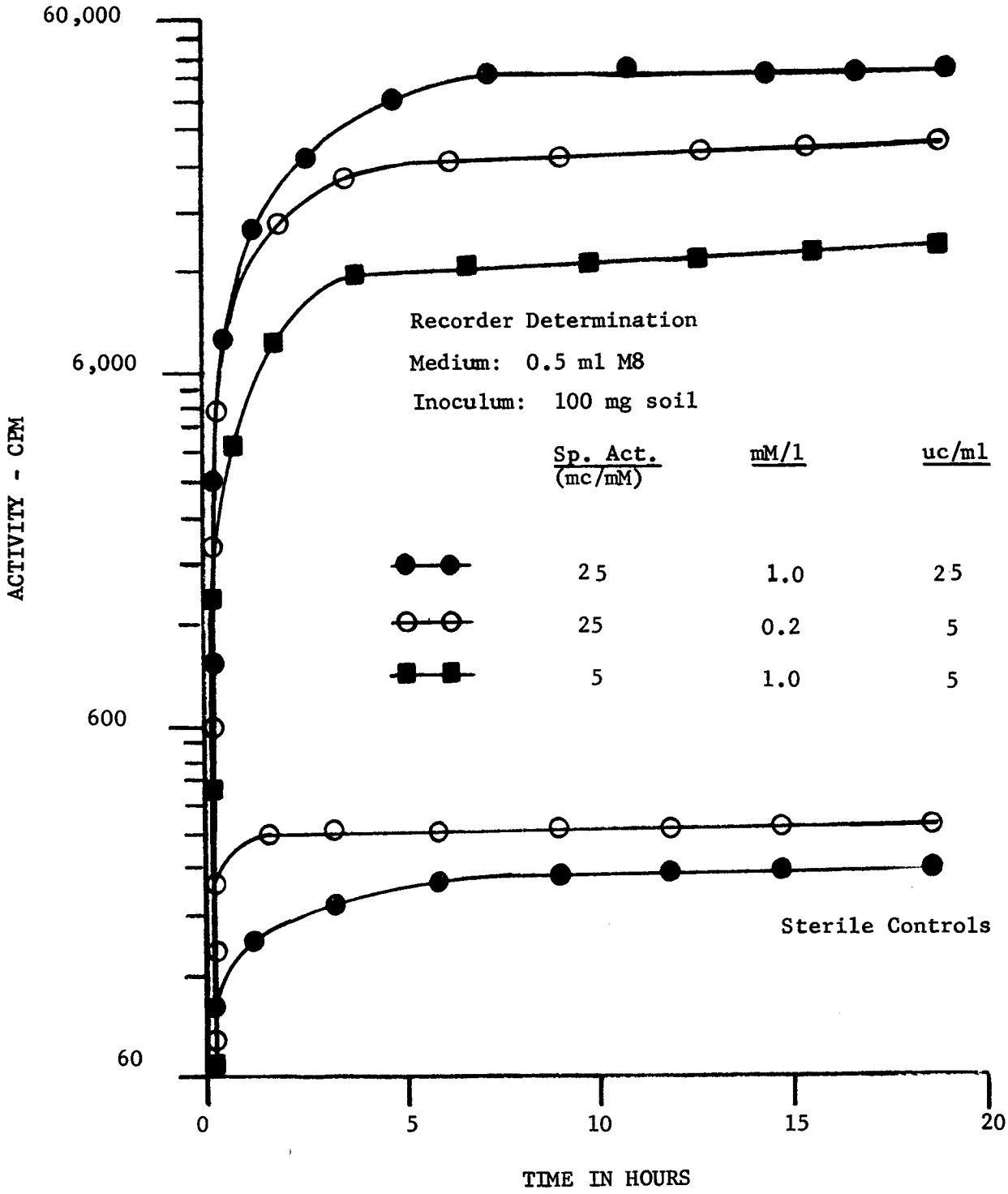
Table III-7

COMBINATIONS TESTED FOR EFFECTS OF INCREASED SPECIFIC ACTIVITY OF GLUCOSE-C^{14}

Combination	Specific Activity mc/mM	mM/l	Radioactivity uc/ml
formate	25.0	0.20	5.00
glucose	4.7	0.20	1.00
		0.40	6.00
formate	25.0	0.20	5.00
glucose	12.0	0.08	1.00
		0.40	6.00
formate	25.0	0.20	5.00
glucose	12.0	0.20	2.50
		0.40	7.50
formate	25.0	0.13	3.30
glucose	4.7	0.14	0.66
lactate	5.0	0.44	2.20
		0.71	6.16
formate	25.0	0.13	3.30
glucose	4.7	0.21	1.00
lactate	5.0	0.44	2.20
		0.78	6.50
formate	25.0	0.13	3.30
glucose	12.0	0.21	2.50
lactate	5.0	0.44	2.20
		0.78	8.00

Figure III-2

EFFECT OF INCREASED SPECIFIC ACTIVITY SODIUM FORMATE-C^{14}



Experiments were conducted on the automated monitoring unit to determine the effect such concentration changes might have. The following C^{14} combinations were examined:

	Sp. Act. (mc/mM)	uc/ml	mM/l
(1) formate	25.0	5	0.20
glucose	4.7	1	0.20
total		6	0.40
(2) formate	25.0	10	0.40
glucose	4.7	2	0.40
total		12	0.80
(3) formate	25.0	6.5	0.26
glucose	4.7	1.3	0.28
lactate	5.0	1.3	0.26
total		9.1	0.80

The first combination, containing the lowest molar and radioactive concentrations, consistently yielded the poorest response. Combinations 2 and 3 responded equally, producing the highest responses, although one contained an additional substrate and the other was at a higher total radioactive level. It is difficult to determine if the greater response from combinations 2 and 3, actually resulted from a two-fold increase in molar concentration or from the greater total radioactivity employed. Nevertheless, it is apparent that, on a molar basis, the formate-glucose-lactate combination did well.

As a result of the various determinations, the basic C^{14} substrate contained the following molar-radioactive concentrations:

	Sp. Act. (mc/mM)	uc/ml	mM/l
Sodium formate	25.0	6.5	0.26
D-glucose-U.L.	4.7	1.3	0.28
DL-sodium lactate-1	5.0	1.3	0.26
total		9.1	0.80

b. New C^{14} Substrates

Another important aspect of the program has been the investigation of new labeled nutrients. The new substrates, in addition to the usual nutritional requirements, need to be chemically and radioactively stable, withstand sterilization temperatures of 135°C for 24 hours, and be compatable with the other medium constituents. The compounds selected for study were: (1) DL-α-alanine-C^{14} (2) glycine-1-C^{14} (3) DL-sodium lactate-1-C^{14} (4) DL-malic acid-3-C^{14} (5) nitrilotriacetic acid-C^{14} (6) DL-tyrosine-1-C^{14} and (7) urea-C^{14}. Each compound was tested in combination with the formate-glucose-C^{14} basic substrates and the results compared to those obtained from the basic C^{14} substrate standard. Those which elicited responses equal to or better than the standard were then considered to be potentially beneficial and warranting possible inclusion into the medium.

In order to obtain comparative results, experimental factors (Set I) were kept at constant levels. These were a radioactive ratio of 5 formate/1 glucose; a molar ratio of 3 formate/1 glucose; and a total concentration of 1.33 mM/1. However, concurrent determinations on the effects of increased specific activities of formate and glucose (Medium Development, Section IIIA), necessitated alterations of the concentration levels. The formate/glucose radioactive ratio remained unaltered; the molar ratio was changed to 1 formate/1 glucose; the total concentration became 0.80 mM/1 (Set II).

The glycine, lactate, malic and urea substrates (Set I) were compared to the formate-glucose standard in combination with the two basic substrates and also as the sole C^{14} substrate. The remaining new substrates (Set II) were compared to the standard in combination only, not individually. A summary of the experimental details is presented on the following page.

Method ----- Automated monitoring and recording system

Medium ----- M8 or M9, 0.5 ml

C^{14} Substrates ----- (Set I) ----- Glycine, Lactate, Malic acid, Urea

(1) Standard	Sp. Act. (mc/mM)	mM/l (Total 1.33)	uc/ml	% (w/v)
formate	5.0	1.00	5.00	0.007
glucose	3.0	0.33	1.00	0.006
(2) New Substrate Combination		(Total 1.32)		
formate	5.0	0.66	3.33	0.004
glucose	3.0	0.22	0.66	0.004
* plus one new substrate	*	0.44 1.32	*	*
* New substrate				
glycine	4.4*	0.44	1.30*	0.003*
or				
lactate	5.0*	0.44	2.20*	0.005*
or				
malic acid	6.8*	0.44	3.00*	0.006*
or				
urea	2.1*	0.44	0.90*	0.003*
(3) New Substrate Concentration - Tested Individually				
glycine	4.4	1.33	4.00	0.010
lactate	5.0	1.33	6.65	0.015
malic acid	6.8	1.33	9.00	0.018
nitrilo-triacetic acid	0.02	1.33	0.50	0.025
urea	2.1	1.33	2.70	0.080

C^{14} Substrates ----- (Set II) ----- Alanine, Tyrosine

(1) Standard	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
formate	25.0	0.40	10.0	0.003
glucose	4.7	0.40 .80	2.0	0.007
(2) New Substrate Combination				
formate	25.0	0.26	6.5	0.002
glucose	4.7	0.28	1.3	0.005
* plus one new substrate	*	0.26 0.80	*	*
* New Substrate				
alanine	7.7*	0.26*	2.0*	0.002*
or				
tyrosine	3.7*	0.26*	1.0*	0.005*

Inocula ----- Minimum of 2 soils and 2 pure cultures/determination

Soils (100 mg/test)	Pure Cultures (0.2 ml/test)
Apple Valley (Calif.)	E. coli
Garden (D. C.)	B. subtilis v. globigii (veg.)
Iron-rich (Orange, Va.)	B. subtilis v. globigii (spores)
Field (Va.)	S. cerevisiae
Metuchen (N. J.)	R. rubrum
Rocky Mountain, Colo.	P. fluorescens
New Brunswick (N. J.)	

Total incubation and collection period ----- Continuous for 18 hours

Results ----- The alanine, nitrilotriacetic acid, malic acid, and urea did not yield responses warranting further investigation. The sodium lactate (tested previously - Annual Report #2), however, continued to produce favorable responses, Figure III-3, and was incorporated into the basal medium, thus containing labeled formate, glucose, and lactate. Responses from the tyrosine, although not very pronounced, were present, particularly in the initial hours. Additional determinations are planned for this compound.

The presence of glycine in the medium also elicited desirable responses, Figure III-4. Consequently, additional determinations were performed, incorporating the new M9 medium containing the formate-glucose-lactate-C^{14} substrates. Experimental details follow.

Method ----- Planchet determination

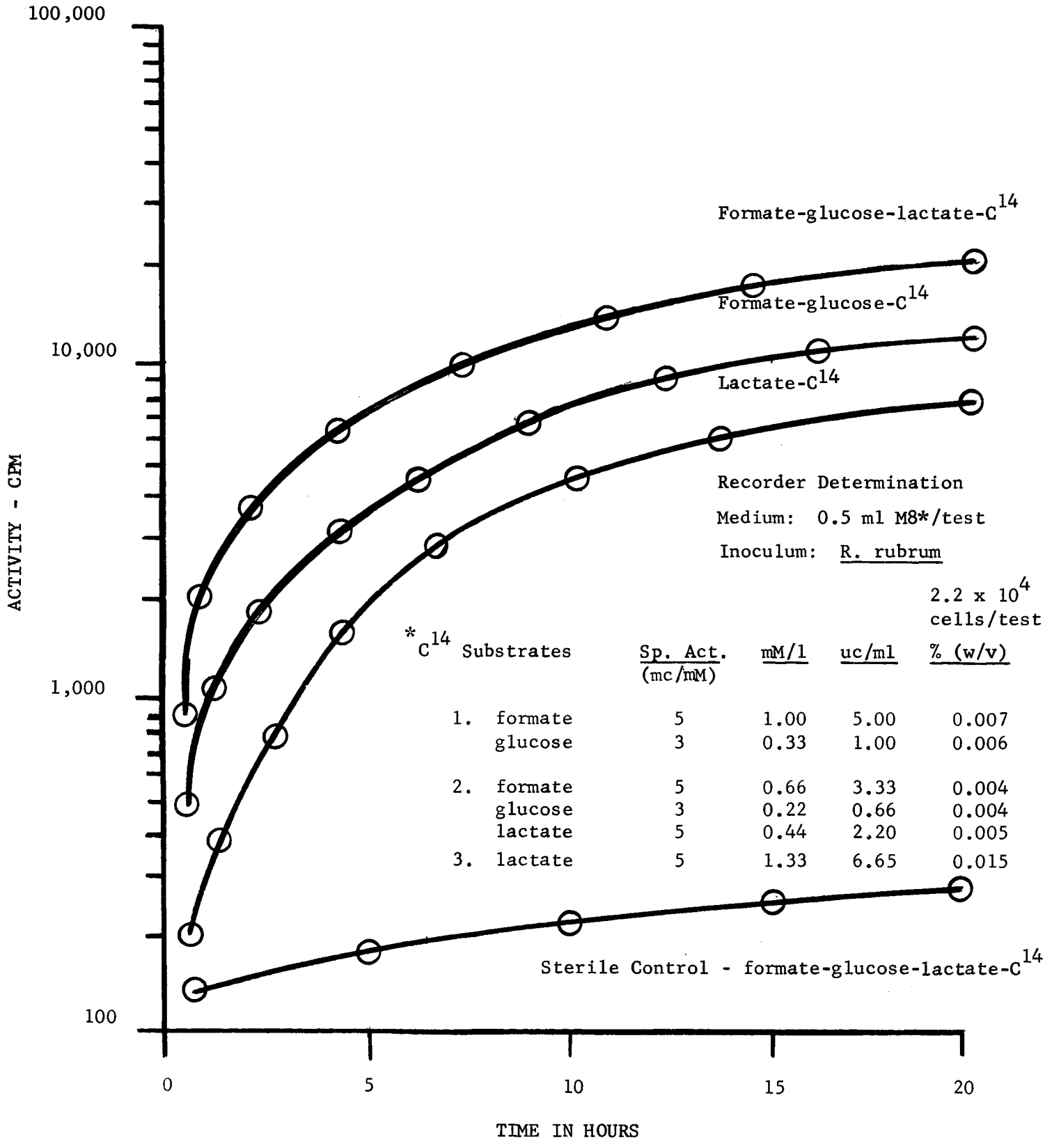
Medium ----- M9, 0.5 ml

C^{14} Substrates -----

	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
(1) Standard				
formate	25.0	0.24	6.0	0.002
glucose	4.7	0.28	1.3	0.005
lactate	5.0	0.26	1.3	0.002
(2) New substrate combination				
formate	25.0	0.24	6.0	0.002
glucose	4.7	0.28	1.3	0.005
lactate	5.0	0.26	1.3	0.002
glycine	4.4	0.22	1.0	0.002

Figure III-3

RESPONSE FROM M8 MEDIA CONTAINING SODIUM LACTATE-C^{14} WITH R. RUBRUM GROWN PHOTOSYNTHETICALLY

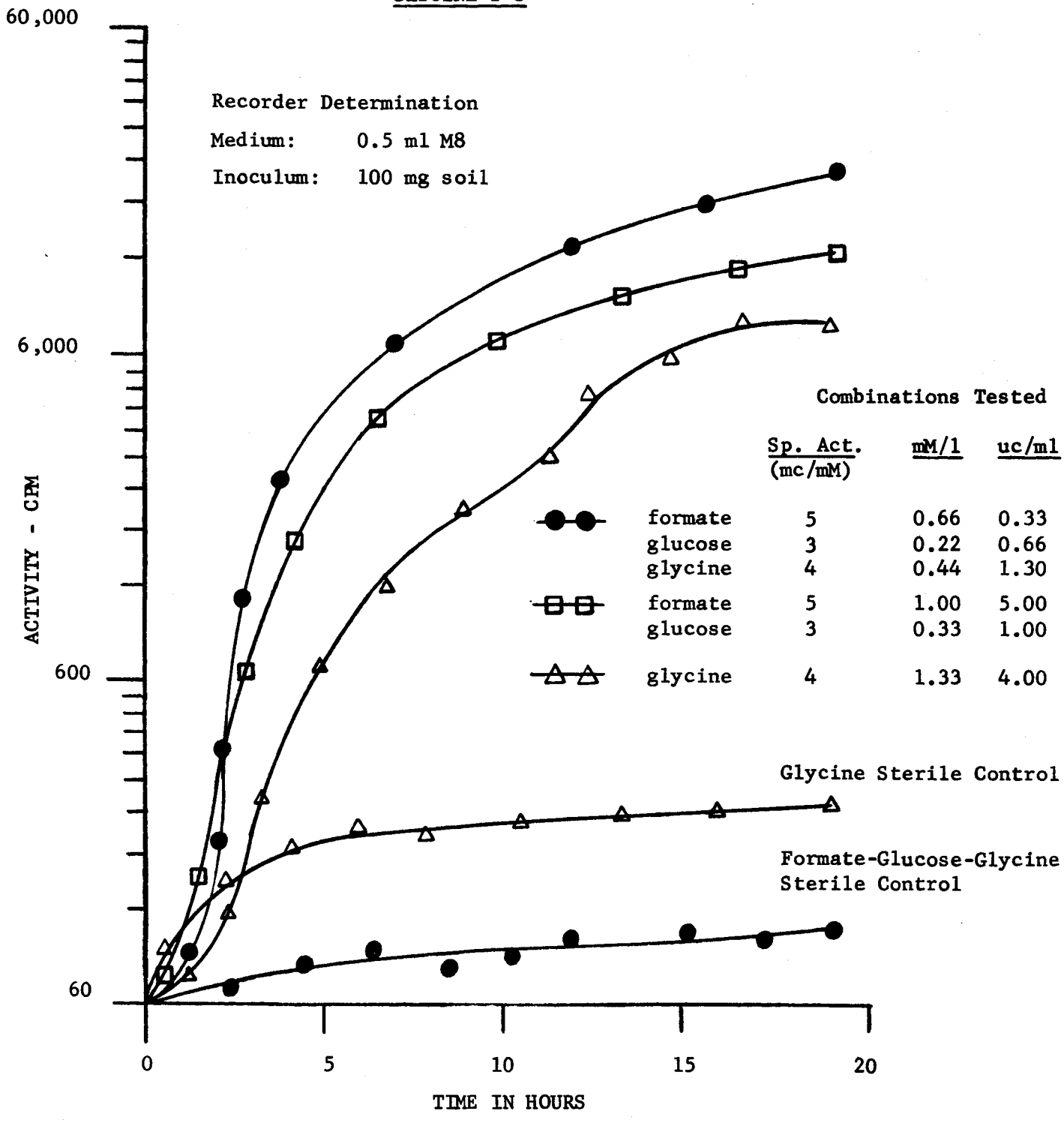


*C^{14} Substrates	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
1. formate	5	1.00	5.00	0.007
glucose	3	0.33	1.00	0.006
2. formate	5	0.66	3.33	0.004
glucose	3	0.22	0.66	0.004
lactate	5	0.44	2.20	0.005
3. lactate	5	1.33	6.65	0.015

Figure III-4

RESPONSE FROM M8 MEDIA CONTAINING

GLYCINE-1-C^{14}



Inocula ----- 100 mg/test
Apple Valley soil
Rocky Mountain soil
Iron-rich soil (Orange, Va.)
California salt soil

Successive Incubation Periods ----- 0.5, 1.0, 2.0, 4.0 hours

$C^{14}O_2$ collection time ----- 15 minutes following each incubation period

Results ----- The responses obtained from the glycine combination were not as advantageous as expected. However, since glycine is considered to be of potential value (a Miller compound) for microorganisms on Mars, and since it was compatable with the basal medium, its incorporation into the medium was deemed advisable.

As a result of the labeled nutrient study a new, more advantageous C^{14} substrate was developed for the basal medium. The constituents are:

C^{14} Substrates -----	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL-sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.4	0.22	1.0	0.002

Results obtained from its use have been most favorable, of any to date, particularly with soil organisms, as illustrated by the field test responses, Figures II-12 to II-15.

The testing of labeled C^{14} compounds for possible inclusion into the basal medium will continue.

c. S^{35} Labeled Substrates

Emphasis has been placed primarily upon carbon-14 substrates throughout most of the program (although a few early studies were performed on sulfur-35 compounds). Now that the medium has become more defined, the use of labels other than carbon-14 has been under reconsideration. Two sulfur-35 compounds were obtained and are presently under investigation. In conjunction with the testing of autotrophic microorganisms, specifically T. thiooxidans and T. novellus, sodium thiosulfate-S^{35} (outer S tagged) and sodium sulfate-S^{35} were selected.

The routine laboratory procedures utilizing C^{14} substrates did not require any modification for the S^{35} compounds since the Gulliver system is capable of detecting the H_2S^{35} evolved. Planchet determinations were performed with variously labeled C^{14} and S^{35} media. The experiment is described and evaluated in the section dealing with responses of test organisms, Section IIIB. The results obtained with sodium thiosulfate-S^{35} in combination with C^{14} labeled substrates as well as the sole labeled substrate were not as promising as expected. However, since only a limited number of determinations have been made to date, definite conclusions regarding utilization of sulfur-35 substrates cannot be made at present. Experiments now in progress are incorporating the sodium sulfate-S^{35} and are also increasing the range of test organisms and basal media.

B. TEST MICROORGANISMS AND RESPONSES

1. Pure Cultures

A set of stock cultures has been obtained and maintained during the course of the space program. The collection was designed to contain a wide range of representative types of microorganisms. As the need arose, additional species were added. At present, there are 36 bacteria, streptomycetes, fungi, and algae in the collection. The organisms include autotrophs, heterotrophs; aerobes, anaerobes; spore formers and non-spore formers. All of the cultures tested by the radioisotope technique have responded positively. The selection of specific cultures from the collection for laboratory experimentation has been dependent upon the purpose and scope of each determination. Usually, preliminary studies will include Gram negative, Gram positive, and yeast cultures. Additional cultures are tested as the study progresses until either sufficient data have been obtained, all of the necessary organisms have been tested, or both.

The listing and general characteristics of the test organisms are presented in Tables III-8 and III-9. Responses obtained from the cultures will be found throughout the report. Table III-10 presents the representative responses of recent additions to the collection. The tabular results are compiled from various planchet determinations.

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The metabolic responses of the autotrophs, Thiobacillus novellus and Thiobacillus thiooxidans were investigated in conjunction with medium development. The autotrophic organisms generally require specific inorganic energy sources; are often incapable of utilizing organic nutrients; and may be inhibited by organic compounds. Facultative autotrophs fortunately combine a few of the heterotrophic characteristics and are

Table III-8

TEST COLLECTION ORGANISMS

Obtained Feb. 1961 - Feb. 1963

Organism	Gram Stain	Oxygen Requirements	Optimum Temperature	Spore Forming	Habitat	Additional Information
Arthrobacter simplex	Gram-pos. rod	Anaerobic	26 - 37°C	No	Soil	Heterotrophic, reduces nitrates to nitrites, utilizes ammonium salts and nitrates for nitrogen
Azotobacter agilis	Gram-neg. rod	Aerobic	25 - 28°C	No	Water and Soil	Heterotrophic, actively fixes atmospheric nitrogen
Azotobacter indicus	Gram-neg. rod	Aerobic	30°C	No	Soil	Heterotrophic, motile, fixes atmospheric nitrogen
Bacterium bibulum	Gram-neg. rod	Aerobic, facultatively anaerobic	20°C	No	Soil	Heterotrophic, produces ammonia
Chlorella pyrenoidosa	-----	------	39°C	--	Fresh water	Photosynthetic, representative algae
Clostridium pasteurianum	Gram-pos. rod	Anaerobic	25°C	Yes	Soil	Heterotrophic, fixes atmospheric nitrogen
Clostridium roseum	Gram-pos. rod	Anaerobic	37°C	Yes	Soil	Heterotrophic, produces ammonia from nitrates nitrites
Clostridium sporogenes	Gram-pos. rod	Anaerobic	37°C	Yes	Soil	Heterotrophic, rapidly reduces nitrates

Table III-8 (Continued)

TEST COLLECTION ORGANISMS

Obtained February, 1961 - February, 1963

Organism	Gram Stain	Oxygen Requirements	Optimum Temperature	Spore Forming	Habitat	Additional Information
Escherichia coli	Gram-neg. rod	Aerobic, facultatively	37°C	No	Intestinal tracts	Heterotrophic
Micrococcus cinnabareus	Gram-variable coccus	Aerobic	25°C	No	Dust	Heterotrophic, reduces nitrates to nitrites
Mycobacterium phlei	Gram-pos. rod	Aerobic	28 - 52°C	No	Soil, dust, and plants	Heterotrophic, non motile
Photobacterium phosphoreum	Gram-neg. coccobacillus	Aerobic, facultatively anaerobic	5 - 10°C	No	Dead fish and sea water	Heterotrophic, psychrophilic, anaerobic fermentation of glucose
Pseudomonas delphinii	Gram-neg. rod	Aerobic	25°C	No	Plants	Heterotrophic, capable of growth at 1°C or less
Pseudomonas fluorescens	Gram-neg. rod	Aerobic	20 - 25°C	No	Soil and water	Heterotrophic, motile, nitrates reduced to nitrites and ammonia
Pseudomonas maculicola	Gram-neg. rod	Aerobic	25°C	No	Plants	Heterotrophic, capable of growth at 0°C
Rhizobium leguminosarium	Gram-neg. rod	Aerobic	25°C	No	Soils in which legumes are grown	Heterotrophic, motile, fixes nitrogen symbiotically
Rhodopseudomonas capsulata	Gram-neg. spheres and rods	Aerobic anaerobic	25°C	No	Stagnant water, mud	Heterotrophic, can grow anaerobically by photosynthesis

Table III-8 (Continued)

TEST COLLECTION ORGANISMS

Obtained February, 1961 - February, 1963

Organism	Gram Stain	Oxygen Requirements	Optimum Temperature	Spore Forming	Habitat	Additional Information
Rhodospirillum rubrum	Gram-neg. spiral	Capable of being strictly anaerobic - photosynthetically; microaerophilic in dark	30 - 37°C	No	Stagnant water and mud	Heterotrophic or phototrophic, can grow anaerobically and photosynthetically
Saccharomyces cerevisiae	Gram-pos.	Aerobic, facultatively anaerobic	30 - 37°C	Yes	Soil, plants	Representative fungus
Streptomyces bobiliae	Gram-pos. mycelium	Aerobic	30 - 37°C	Yes	Soil	Heterophic, forms branching filaments and hyphae similar to fungi
Streptomyces fradiae	Gram-pos. mycelium	Aerobic	25 - 30°C	Yes	Soil	
Thiobacillus novellus	Gram-neg. rod	Aerobic	28 - 30°C	-----	Soil	Facultatively autotrophic, oxidizes thiosulfate to sulfate and sulfuric acid
Thiobacillus thiooxidans	Gram-neg. rod	Aerobic	28 - 30°C	-----	Soil	Strictly autotrophic, oxidizes elemental sulfur and thiosulfate to sulfuric acid
Xanthomonas beticola	Gram-neg. rod	Aerobic	29°C	No	Plants	Heterotrophic, capable of growth from 1.5 to 39°C, high salt tolerance

Table III-9

NEW ORGANISMS ADDED TO TEST COLLECTION

February, 1963 - February, 1964

Organism	Gram Stain	Oxygen Requirements	Temperature	Forming	Habitat	Additional Information
Aerobacter aerogenes	Gram-neg. rod	Aerobic, facultatively anaerobic	30°C	----	Widely distributed in nature	Heterotrophic, non motile
Bacillus subtilis var. globigii 1. vegative 2. spore suspension	Gram-neg. rod	Aerobic	28 - 40°C	Yes	Soil	Heterotrophic, motile, produces extremely resistant spores
Clostridium perfringens	Gram-pos. rod	Strictly anaerobic	35 - 37°C	Yes	Sewage, soil	Heterotrophic, non motile
Ferrobacillus ferrooxidans	Gram-neg. rod	Aerobic	15 - 20°C	----	Coal, soil, natural or artifact ferrous deposits	Strictly autotrophic, motile, oxidizes ferrous iron to ferric iron - optimum reaction pH 3.5
Lactobacillus plantarum	Gram-pos. rod	Microaero-philic	30°C	-----	Widely distributed in nature	Heterotrophic, non motile, produces little or no CO_2 from sugar fermentation
Serratia marcescens	Gram-neg.	Aerobic, facultatively anaerobic	25 - 30°C	No	Water, soil, milk, food	Heterotrophic, motile

Table III-9 (Continued)

NEW ORGANISMS ADDED TO TEST COLLECTION

February, 1963 - February, 1964

Organism	Gram Stain	Oxygen Requirements	Optimum Temperature	Spore Forming	Habitat	Information
Leptothrix cholodnii (strains 1, 19, 99)	Gram-neg. rod	Aerobic	28°C	-----	Water containing ferrous iron	Heterotrophic, true iron bacterium
Leptothrix discophora (strains 35, 37, 63)	Gram-neg. rod	Aerobic	28°C	-----	Water containing ferrous iron	Heterotrophic, true iron bacterium
Leptothrix lopholea (strain 76)	Gram-neg. rod	Aerobic	28°C	-----	Water containing ferrous iron	Heterotrophic, true iron bacterium
Leptothrix pseudo-ochracea (strain 41V)	Gram-neg. rod	Aerobic	28°C	-----	Water containing ferrous iron	Heterotrophic, true iron bacterium
Sphaerotilus natans (strains 3, 52)	Gram-neg. rod	Aerobic	28°C	-----	Water contaminated with sewage; paper, and dairy industrial wastes	Heterotrophic, motile sheathed bacterium, may or may not be true iron bacterium

Table III-10

TYPICAL RESPONSES OF NEW TEST COLLECTION MICROORGANISMS
PLANCHET DETERMINATION

Inoculum (0.1 ml)	Medium (0.5 ml)	Average Net Radioactivity - CPM 3-Hour Incubation Period plus 15 Minute $C^{14}O_2$ Collection
Bacillus subtilis var. globigii-veg.	M9**	50,000
Serratia marcescens	M9*	73,600
Lactobacillus plantarum	M5*	200
Sphaerotilis natans strain 52	M9*	15,800
Leptothrix cholodnii strain 1	M9*	2,200
Leptothrix pseudo-ochraceae strain 41V	M9*	200
Leptothrix diacophora strain 37	M9*	1,900

* C^{14} Substrates - formate-glucose-lactate-glycine

** C^{14} Substrates - formate-glucose-lactate

therefore not as limited as strict autotrophs. The Thiobacilli selected for study represent a strict autotroph, T. thiooxidans and a facultative autotroph, T. novellus. The organisms were tested in M9, a relatively simple basal medium (Table III-1). However, when the labeled C^{14} substrates (formate-glucose-lactate-glycine) are incorporated, the medium, in relation to autotrophic requirements, becomes organically complex. To overcome this, sodium thiosulfate-S^{35}, a compound which both organisms are capable of oxidizing, was incorporated as the sole labeled substrate in one set of experiments. It was also used in combination with the four C^{14} substrates. The responses, shown in Table III-11, were most interesting. Both organisms responded at a high metabolic rate to the M9 medium containing the four C^{14} substrates and at an equally high rate to the combined $C^{14}S^{35}$ medium. The poorest response by both cultures was to the M9 containing only the labeled thiosulfate. Being a facultative autotroph, the T. novellus responses to the more complex media were expected. Those of the strict autotroph, however, were surprisingly high with the M9 medium containing the complex labeled substrates. The results of the experiment were most encouraging. Not only were rapid and high responses obtained, but the autotrophs were apparently able to utilize the "all purpose" C^{14} labeled M9 medium.

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Lactobacillus plantarum was obtained for the test collection to represent a group of bacteria which are reported (Bergey's Manual of Determinative Bacteriology) to produce little or no carbon dioxide from sugar fermentation. It seemed possible that other carbon sources in the medium might be utilized sufficiently to produce a measurable response, and, also, that, if any $C^{14}O_2$ was produced from the glucose, the sensitivity of the method might detect it.

Planchet determinations were performed utilizing the simple M9 medium and the nutritionally rich M5 medium. Both media contained formate-glucose-lactate-glycine-C^{14} substrates. Net averages of 90 cpm in 0.5 hours; 160 cpm in 1.0 hour and 150 cpm in 4.0 hours were detected from the M9. The M5 produced approximately 100 cpm in 0.5 hours, 120 cpm in 1.0 hour and 260 cpm in 4.0 hours from the 3×10^7 cells used as inocula. The initial responses from both media were similar; an increase was obtained with the complex medium upon longer incubation. Taking into consideration the number of cells in the inocula, the responses were relatively low. However, it was possible to detect metabolism, demonstrating, that $C^{14}O_2$ was produced from the substrates present.

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The possible presence of iron salts on the surface of Mars (it has been speculated that the redness of the planet is due to iron) prompted an environmental field testing of the iron-rich soil at Orange, Virginia (Field Test Figure II-11). Numerous laboratory determinations on soil collected at the site were also made. Since all of these responses were representative of heterogenous populations, additional responses from pure cultures of iron microorganisms were desired. The following ten cultures of various iron bacteria were added to the test collection.

Sphaerotilis natans (strains 3, 52)
Leptothrix discophora (strains 35, 37, 63)
Leptothrix cholodnii (strains 1, 19, 99)
Leptothrix pseudo-ochracea (strain 41V)

The Sphaerotilis species may or may not be true iron bacteria; the Leptothrix are considered true iron organisms. Additional characteristics are presented in Table III-10.

Planchet determinations have been performed on four of the iron cultures; 0.5 ml of M9-formate-glucose-lactate-glycine-C^{14} medium was seeded with 0.1 ml of undiluted inoculum. $C^{14}O_2$ was collected for 15 minutes following selected, successive, incubation periods. Plate counts were made on all of the inocula. All four organisms responded positively (Table III-12), although the response from L. pseudo-ochracea indicated a decrease of metabolism as time progressed (this bacterium normally responds poorly to organic nutrients). The plate counts results were very inconclusive, indicating the difficulty of growing these organisms. Only the L. cholodnii produced a reportable number of colonies. Approximately 23,100 cells were present in the 0.1 ml inoculum of that culture.

The remaining strains will be tested similarly, although it is apparent from the present results that iron bacteria can produce good measurable responses from the basal medium.

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Bacillus subtilis var. globigii was obtained for the collection because of its ability to produce extremely resistant spores. Spore suspensions were prepared and maintained as stock. Both the vegetative cells and the spores were used as inocula for various experiments. Relatively good positive responses were elicited from the vegetative cultures; however, the spore suspensions responded very poorly or not at all. Most probably germination did not occur, thus preventing utilization of the labeled constituents of the triple tagged M8 medium. The spore suspensions will be tested in the various media under development as well as in media tailored for germination.

Table III-12

RESPONSES OF IRON BACTERIA TO M9* MEDIUM
PLANCHET DETERMINATION

Organism	Average Net Radioactivity - CPM Successive Incubation Periods**			
	45 min.	90 min.	180 min.	5 hrs. 15 min.
Sphaerotilis natans strain 52	3,162	9,573	15,886	23,521
Leptothrix cholodnii strain 1	640	1,157	2,212	3,735
Leptothrix discophora strain 37	535	771	1,938	2,610
Leptothrix pseudo-ochracea strain 41V	337	318	201	181

* M9 C^{14} Substrates	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL-sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.4	0.22	1.0	0.002

** Each incubation period includes a 15 minute $Ba(OH)_2$ collection period.

Rhodospirillum rubrum is the most versatile organism of the test collection. It has been cultured aerobically and anaerobically; non-photosynthetically and photosynthetically; and in various combinations. Anaerobic and aerobic determinations were made photosynthetically. (The specially designed anaerobic cabinet was not completed at the time. Brewer Jars were used for the anaerobic determinations.) After four hours of incubation, 2.5×10^5 cells produced 917 cpm aerobically, and 300 cpm anaerobically. Additional photosynthetic determinations were made using the specially designed 'photosynthetic' chambers of the algae experiments (Section IIIE). Figure III-3 illustrates the photosynthetic response.

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The microorganisms comprising the test collection have been of great value collectively and as specific representatives of various species. The diversified metabolic requirements of the organisms have provided a wide microbial testing range especially necessary for medium development. All of the cultures have responded positively in varying degrees with the exception of a spore suspension of B. subtilis var. globigii responded negatively or very poorly. New cultures will be added to the collection as the need arises; the existing cultures will continue to provide inocula for the various investigations.

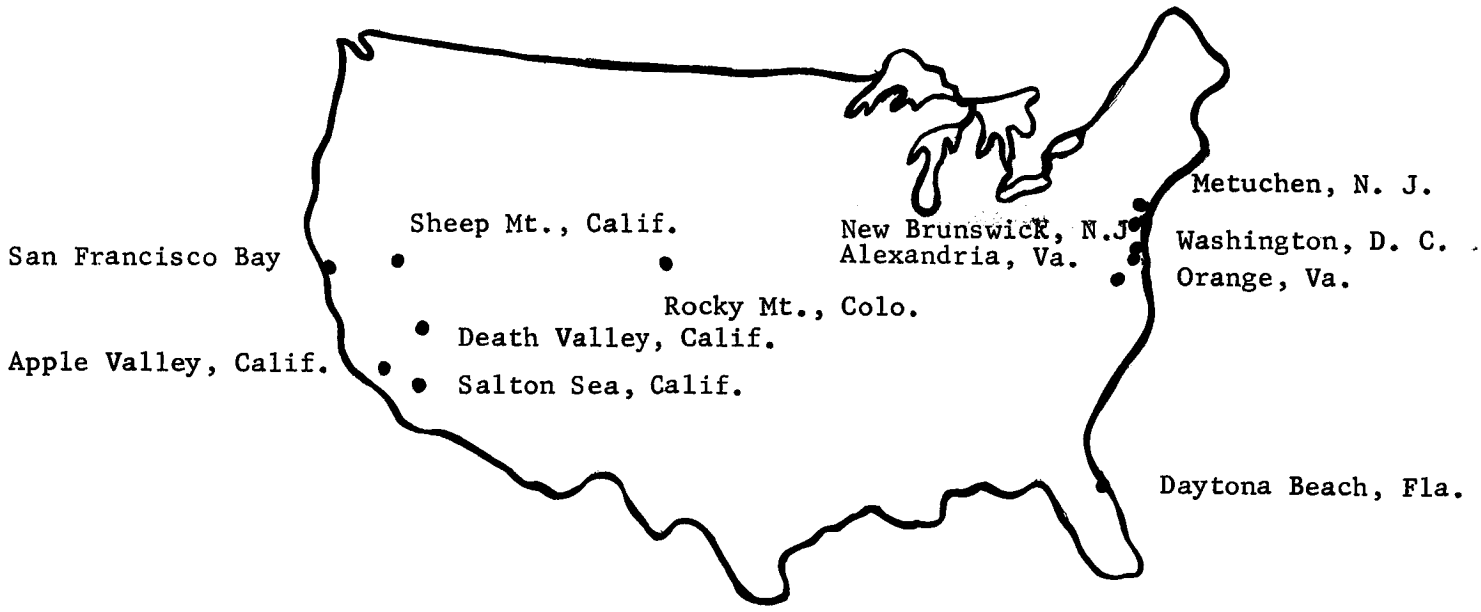
Figure III-5

SOILS

Salton Sea, California

Figure III-6

GEOGRAPHICAL AREAS REPRESENTED IN SOIL-TEST COLLECTION



2. Soils

An important and essential phase of the biological investigation is the soil testing program. Medium development is partly dependent and field trial results wholly dependent upon the responses obtained from soil microflora. As a result, numerous soil organisms are contained in the test organism collection. In addition to the soils obtained locally or by staff members on travel, soil samples were collected aseptically at all field testing areas, permitting subsequent laboratory comparisons to the field test results as well as providing new test material. Figure III-6 illustrates the various locations in the U.S.A. which were sampled.

The twelve soils from the collection which have been the basis for many diversified laboratory tests are listed and briefly described below.

(1) Garden soil from Washington, D. C. A typical local soil selected from an area upon which a field test was conducted in 1962.

(2) Desert soil from Apple Valley, California. A sandy, dry soil containing little organic matter.

(3) Sand from the Mojave Desert, Death Valley, California, (Figure II-10) a field testing site. Analysis of the soil is presented in Table II-2.

(4) Saline soil (Figure III-5) from the Salton Sea flats, California; field testing site. Soil analysis - Table II-2.

(5) Rocks and pebbles - Sheep Mountain, White Mountain range, California. This sample was obtained from the barren, very rocky (Frontispiece), treeless, mountain top (elevation - 12,000 feet) site of the first extreme environment field test. Soil analysis - Table II-2.

(6) Saline soil from the edge of San Francisco Bay near San Carlos, California. The area, near a salt refinery, contains a very high surface salt content.

(7) Mountain tundra soil from the 12,000 foot level of the Rocky Mountains, Colorado.

(8) Saline sand obtained from the edge of the Atlantic Ocean near Daytona Beach, Florida.

(9) A forest type soil from Metuchen, New Jersey.

(10) Field soil from New Brunswick, New Jersey (Rutgers).

(11) Field soil from the sailing marina near Alexandria, Virginia, a field testing site (1962).

(12) Iron-rich soil from the Piedmont Experimental Station, Orange, Virginia. This soil, containing approximately 15% iron oxides, was obtained during a field trial.

A general screening procedure which includes colony counts, automated recorder determinations and planchet determinations, is carried out with each soil. Table III-13 presents the numbers of microorganisms present in test soil as determined on two media; tryptone-glucose agar, a general culturing medium, and Sabouraud agar, for the cultivation of fungi. Figure III-7 illustrates typical soil responses obtained on the automated recorder. For comparative purposes, a three hour incubation period was selected to depict the average responses obtained from soils in planchet studies - Table III-14. The responses obtained from the soils have all been positive, generally quite rapid and high.

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Additional determinations were performed on the saline soils. The soils, from the edge of San Francisco Bay (California) near a salt refining plant and from the Atlantic Ocean (Daytona Beach, Florida)

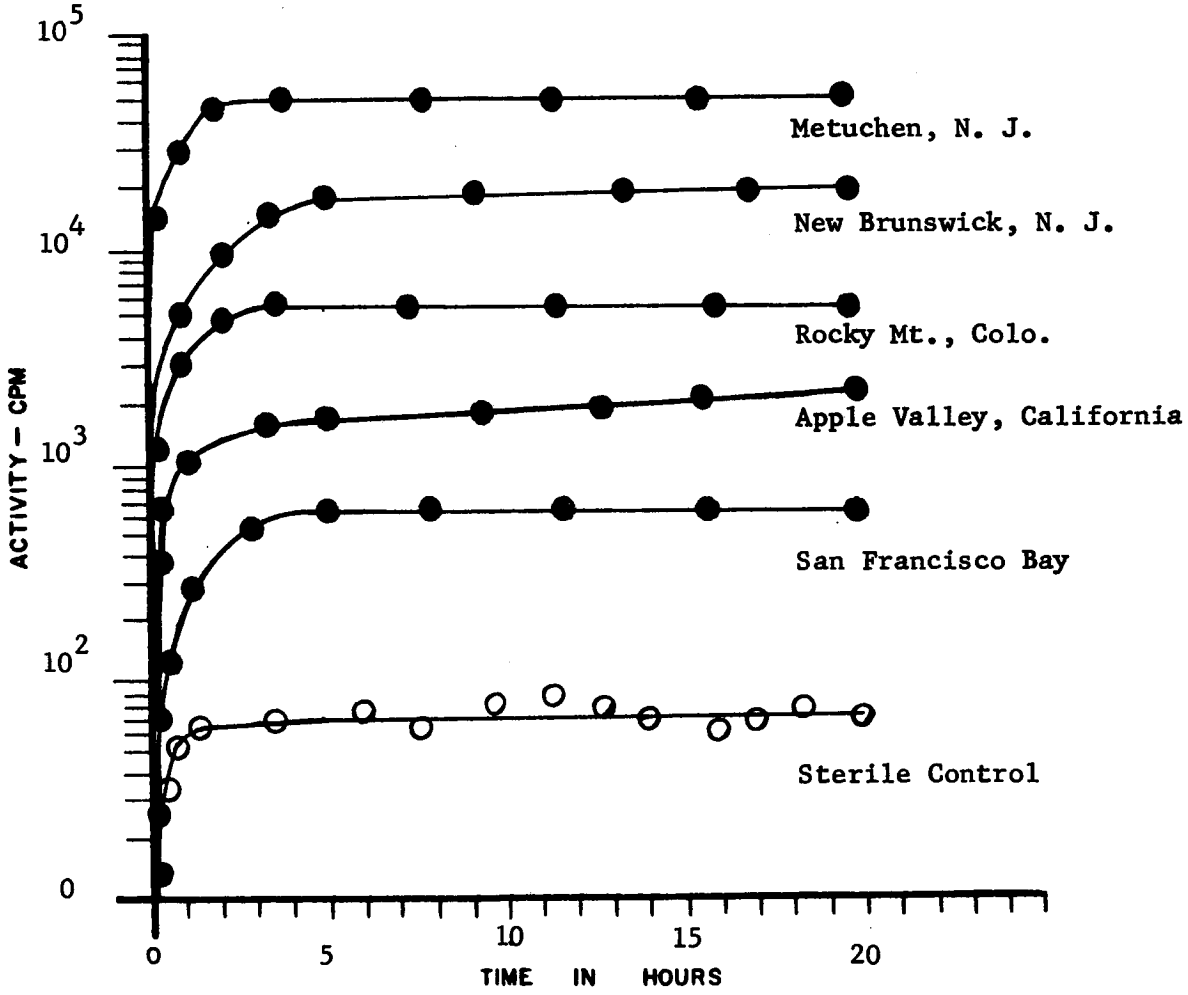
Table III-13

NUMBERS OF MICROORGANISMS ISOLATED FROM TEST SOILS

SOIL	LOCATION	ORGANISM/100 mg SOIL	
		Tryptone-glucose agar	Sabouraud agar
Garden	Washington, D. C.	112,500	365
Desert	Apple Valley, Calif.	49,000	50
Sand	Death Valley, Calif.	1,950	1
	algae	3,950	
Saline	Salton Sea, Calif.	52,000	200
Rock	Sheep Mt., Calif.	28,000	16
Saline	San Francisco Bay, Calif.	1,700	0
Mountain	Rocky Mountain, Colo.	5,950,000	86,000
Forest	Metuchen, N. J.	81,000	20,500
Field	New Brunswick, N. J.	608,000	6,750
Field	Alexandria, Va.	88,500	230
Iron-rich	Orange, Va.	148,000	19,500
	algae	7,500	

Figure III-7

RESPONSES FROM SOILS TO M8 MEDIUM



Recorder Determination

Inoculum: 100 mg soil

Medium: 0.5 ml M8/test

C^{14} Substrates: formate-glucose-lactate

Table III-14

TYPICAL RESPONSES FROM TEST SOILS
PLANCHET DETERMINATION

SOIL (100 mg)	LOCATION	MEDIUM* (0.5 ml)	AVERAGE NET RADIOACTIVITY** - CPM
Garden	Washington, D. C.	$M8^3$	15,000
Field	Alexandria, Va.	$M8^3$	3,700
Desert	Apple Valley, Calif.	$M9^3$	2,800
Saline-sand	Daytona Beach, Fla.	$M9^3$	500
Forest	Metuchen, N. J.	$M9^3$	34,000
Sand	Death Valley, Calif.	$M9^4$	500
Saline	Salton Sea, Calif.	$M9^4$	21,578
Rock	Sheep Mt., Calif.	$M9^4$	13,392
Saline	San Francisco Bay, Calif.	$M9^4$	900
Mountain	Rocky Mt., Colo.	$M9^4$	28,000
Iron-rich	Orange, Va.	$M9^4$	36,000

* Medium
$M8^3$ - M8 plus formate-glucose-lactate-C^{14}
$M9^3$ - M9 plus formate-glucose-lactate-C^{14}
$M9^4$ - M9 plus formate-glucose-lactate-glycine-C^{14}

** Three hour incubation period followed by 15 minute $C^{14}O_2$ collection period.

Additional determinations were performed on the saline soils. The soils, from the edge of San Francisco Bay (California) near a salt refining plant and from the Atlantic Ocean (Daytona Beach, Florida) contained a high percentage of salt. Responses to the basal medium by the halophilic organisms present in these soils were of particular interest since there is speculation that the surface of Mars might be of high salt concentration.

To adapt the halophilic organisms found in these soils to high salinity laboratory environments, they were transferred to special halophilic broths of successively increased salt concentrations. The constituents of this medium* are presented below:

NaCl	5%, 7%, 10%, 18%, 20%
$MgCl_2 \cdot 6H_2O$	4.00%
KCl	0.20%
$CaCl_2$	0.01%
Tryptone	0.50%
Yeast Extract	0.50%

pH 7.0 - 7.2

Approximately seven to ten days of incubation were required to obtain good growth in each of the successive salt cultures. When growth was present in the final 20% salt medium, planchet determinations were performed: 0.5 ml of the M9-formate-glucose-lactate-glycine-C^{14} was seeded with 0.1 ml of the saline culture. The metabolic $C^{14}O_2$ was collected for 15 minutes following three successive incubation periods. The results, presented on the next page, were very encouraging.

*Suggested by Dr. L. Hockstein, Ames Research Center, Palo Alto, Calif.

SALINE-SOIL	AVERAGE NET RADIOACTIVITY - CPM Incubation Periods (hours)		
	0.75	3.25	5.25
San Francisco Bay	331	949	2,782
Daytona Beach	65	505	1,952

Plate counts were made on 20% halophilic agar, but, after two weeks of incubation, still showed no growth. The responses from the halophilic organisms in soil in M9 medium, although not very high, were excellent. Not only was metabolism present in 45 minutes, but growth was obtained.

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During the California field tests, several in situ determinations were made (Section IIB5). The $C^{14}O_2$ was collected for one hour by wet $Ba(OH)_2$ pads in planchets placed directly on the soil which had been moistened with labeled M9 medium. The responses obtained were rapid and very high (Table II-1), indicating the feasibility of a new 'Gulliver' approach for life detection. Soil was collected from the field test sites and brought back to the laboratory for further determinations. The possibility existed that the responses which had been obtained in the field might have been the result of non-metabolic $C^{14}O_2$ evolution. The two California soils, Death Valley and Salton Sea, as well as three additional test-collection soils were sterilized by autoclaving on four consecutive days. Following sterility checks, the soils were placed in planchets and moistened with 0.25 ml of the labeled M9 medium. Collection pads were put in place immediately, for a one hour incubation-collection period. This set was incubated at room temperature, 27°C; a second set was allowed to incubate at room temperature for two hours and was then placed in a 35°C incubator for a one hour collection. The results are given on the next page.

SOIL (Sterilized)	ONE HOUR INCUBATION-COLLECTION 27°C	ONE HOUR INCUBATION-COLLECTION 35°C FOLLOWING 2 HOUR 27°C INCUBATION
	AVERAGE NET RADIOACTIVITY - CPM	
Death Valley	0	0
Salton Sea	0	0
Sheep Mountain	0	0
Iron-rich (Orange, Va.)	52	176
Metuchen, N. J.	5,520	6,818
(Media Control)	116	152

Two of the soils produced non-metabolic activity, the others, including the two soils used for the in situ determinations did not yield activity above the control level. A repeat determination on the Metuchen soil produced similar high responses. During this test, pH values were determined. The medium alone gave a pH of 7; the medium plus the sterilized Metuchen soil was 5. Consequently, pH values were obtained on all five sterilized soils with the following results.

SOIL	pH
Death Valley	7
Salton Sea	7
Sheep Mountain	8
Iron-rich (Orange, Va.)	5
Metuchen, N. J.	5

In addition, the labeled M9 medium was examined for non-metabolic $C^{14}O_2$ evolution at low pH values. The medium (pH 7) was adjusted with 1 N HCl to pH 4 and pH 5. A one hour $C^{14}O_2$ incubation-collection was made. The pH 4 medium produced 16,151 cpm; the pH 5 medium yielded 7,819 cpm;

verifying experimentally (a prior literature survey indicated that some of the radioactive substrates in use would break down at low pH values) that non-metabolic $C^{14}O_2$ production increased with progressively decreased pH values.

Correlation of the experiments suggested that soils of a low pH, when placed in the neutral M9 medium would produce non-metabolic $C^{14}O_2$. (The iron-rich soil, pH 5, which produced a relatively low non-metabolic activity is presently under study.) However, the in situ results obtained in the California field tests were metabolic, as both of the soils registered a neutral pH.

This problem posed to the Gulliver experiment may be met in three ways. First, pH adjustment of the inoculated medium might be made. This would significantly increase the complexity of the instrument. Secondly, a strong buffering capacity could be built into the medium. This might impose an unnatural pH on Martian organisms and runs counter to the ambient environment philosophy of the experiment. Thirdly, the antimetabolite control may be relied upon to distinguish this chemically evolved gas from metabolic evolution. This would require no complicating alteration to the experiment and is, hence, the most desirable solution. Moreover, to distinguish between chemical and biological generation of gas is the whole purpose of the antimetabolite. Therefore, the pH effect represents one specific problem for which the experimental control was designed.

3. Soil Isolates

An important source of inocula used in laboratory determinations and, of course, the sole inocula in the field trials has been soils. These soils, coming from widely varying environments - Figure III-6

have all yielded positive, and usually, rapid responses. The possibility exists that a small number of metabolically active species, common to numerous soils, are producing all of the responses. In other words, are the responses obtained from soil inocula representative of the collective population of each specific soil or are they the result of a narrow range of species predominantly present in a wide variety of soils.

A screening program was initiated to examine the individual microbial genera of the test soils. Approximately 100 mg of each soil were suspended and shaken in five ml of sterile buffered water. Dilutions of 10^{-1}, 10^{-2}, 10^{-3} were plated for isolation, in triplicate, on Difco Sabouraud Agar containing Chloromycetin and on Difco Tryptone-glucose Agar containing Fungizone. Following incubation at room temperature, the colonies appearing most frequently were selected and subcultured until pure cultures were obtained. The gross morphological characteristics were recorded and gram stains made. Planchet determinations with accompanying plate counts were then performed on each of the isolates.

Five soils were screened and the following different isolates obtained:

1. Twenty-two bacteria, five appearing in more than one soil.
2. Sixteen streptomycetes, one appearing in two of the soils.
3. Two Nocardia
4. One yeast
5. One alga
6. Twenty-two fungi, two of which were isolated from three of the soils and three similar fungi from two of the soils.

Of these, 12 bacteria and one streptomycete have been tested to date. One automatic recorder determination was also performed on a bacterial

isolate and is presented in Figure III-8. In order to supply some degree of natural soil nutrients to the isolates, 100 mg of sterile soil was placed in each of the incubation planchets. Initially, the planchet determinations did not incorporate the sterile soil, but a subsequent study (results presented below) indicated that the factors supplied by the soil were beneficial, particularly with the use of the very simple M8 culturing medium. (Shortly thereafter, soil extract was added to the M8 medium, Medium Development - Section IIIA-1).

Soil Isolate	Net Radioactivity - CPM Successive Incubation Period			Cells/test	CPM/cell (x 10^{-4})
	45 min	90 min	180 min		
50P with soil	12,742	15,466	36,124	6 x 10^4	5470
without soil	9,266	14,089	15,626	4 x 10^4	3906
5U with soil	17,539	23,055	43,340	7 x 10^5	619
without soil	10,599	13,045	12,937	8 x 10^5	158

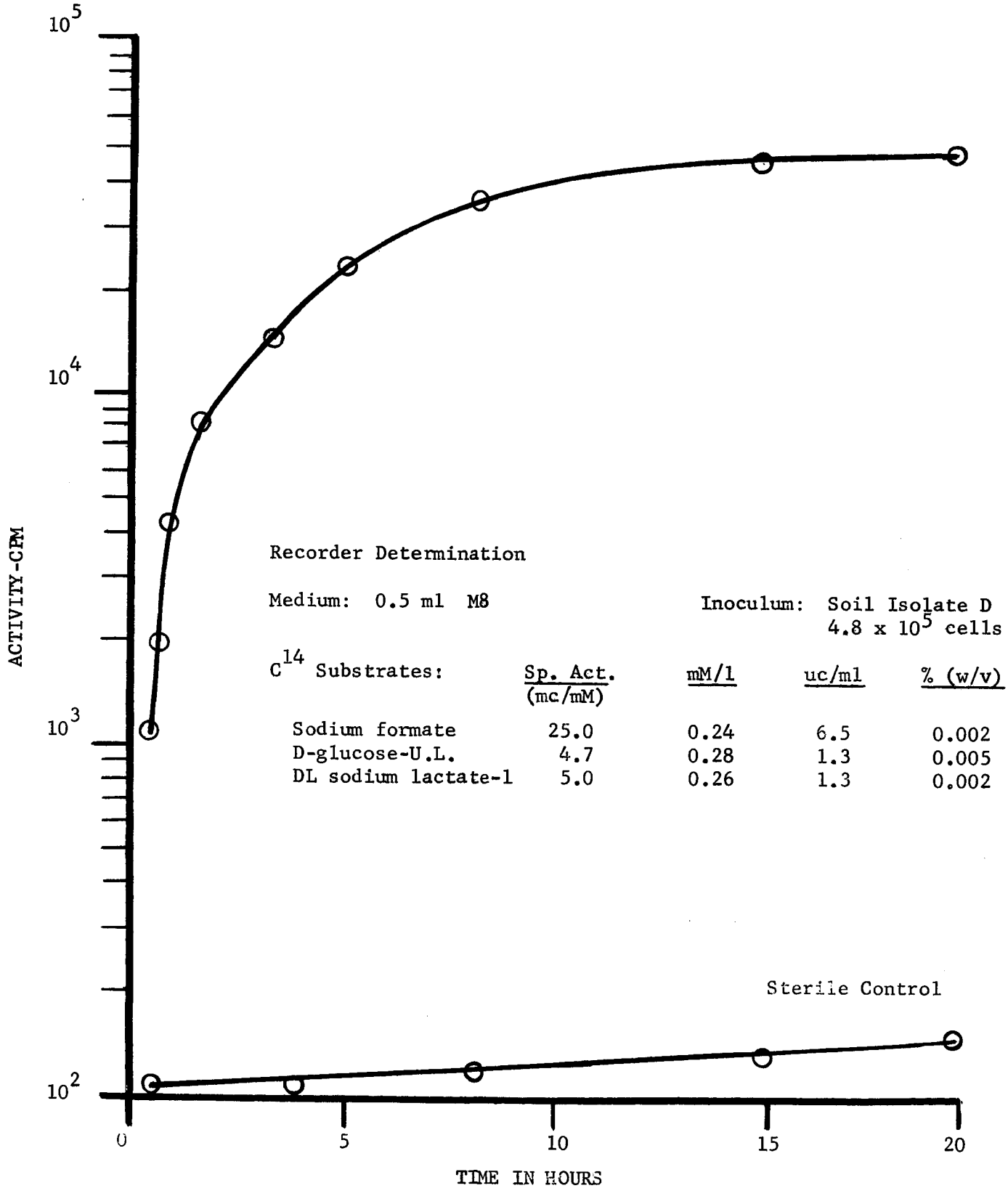
As a result, the routine planchet determination on an isolate incorporated 100 mg sterile soil, 0.5 ml of M8-formate-glucose-lactate-C^{14} medium and 0.1 ml of a direct or diluted broth culture inoculum. The general characteristics and responses of the bacterial soil isolates are presented in Table III-15 and III-16.

Of particular interest were the first responses obtained from mycelial fungi-field and garden soil Isolates E and F. In this early preliminary study the fungi were cultured in M8-formate-glucose-C^{14} medium. The results of recorder determinations were:

	Cells/test	Average Net Radioactivity - CPM Successive Incubation Period (hours)				
		0.5	1	2	4	8
Isolate E	-----	130	210	400	750	1500
Isolate F	58,400	50	120	270	1120	4590

Figure III-8

RESPONSE OF SOIL ISOLATE D TO M8 MEDIUM



Recorder Determination

Medium: 0.5 ml M8

Inoculum: Soil Isolate D 4.8×10^5 cells

C^{14} Substrates:	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.24	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL sodium lactate-1	5.0	0.26	1.3	0.002

Plate counts were made, but only those for Isolate F were obtained. This fungus grew initially as discrete white mycelial colonies, enabling an approximate reading to be made. Isolate E, a penicillum-like fungus, failed to grow on the plates, although heavy growth did occur on slants and in broth cultures. The positive responses obtained from these prominant representatives of soil microflora were very encouraging. Approximately 20 fungi, isolated from the five soils under study, will be tested in the forthcoming year.

A complete comparative evaluation cannot be made until all of the isolates are tested. A few response patterns, however, are becoming apparent. Four sets of isolates from different soil sources have given similar cpm/cell values - 2 AB and 1 AB, 2 G and 3 B, 5 U and 3 C, 1 E and 3 G. Of these, morphologically (grossly), microscopically, and metabolically, 2 G and 3 B appear to be the same organism. 1 E and 3 G also appear to be identical. Two other groups of isolates appeared to be morphologically and microscopically alike, but did not respond similarly. This may be due to the difficulties encountered in obtaining accurate cell numbers.

The study will be continued until all of the soil isolates have been examined.

Table III-15

GENERAL CHARACTERISTICS OF TESTED SOIL ISOLATES

Isolate	Soil Source	Gram Stain	Colony Description
1 AB	Rutgers, N. J.	Gram-positive rod	Yellow, mucoid, translucent, changing to white
1 C	Rutgers, N. J.	Gram-negative rod	White, mucoid
1 E	Rutgers, N. J.	Gram-positive rod	White, flat, feathery, spreading, dry, dull
1 F	Rutgers, N. J.	Gram-positive rod	Small, white, mucoid, changing to brown
2 AB	Rocky Mt., Colo.	Gram-negative rod, spores	Flat, round, dry, white irregular edges
2 D	Rocky Mt., Colo.	Gram-positive rod	Flat, white, surrounded by halo
2 G	Rocky Mt., Colo.	Gram-negative rod	Flat, white, transparent
3 A	Apple Valley, Calif.	Gram-positive filaments Streptomycete	Raised, round, hard, amber-yellow, 2/3 growth beneath agar surface
3 B	Apple Valley, Calif.	Gram-negative rod	Flat, white, transparent
3 C	Apple Valley, Calif.	Gram-positive rod	Small, raised, shiny, white, translucent
3 G	Apple Valley, Calif.	Gram-positive rod	Dry, dull, feathery, white, spreading, flat
50 P	Iron-rich Orange, Va.	Gram-positive rod becoming gram-negative with spores	Finely - spreading, feathery, white
5 U	Iron-rich Orange, Va.	Variable	Flat, round, shiny, white becoming dry, dull, wrinkled, brown

Table III-16

RESPONSES OF SOIL ISOLATES IN M8* MEDIUM
PLANCHET DETERMINATION**

Soil	Soil Isolate	Average Net Radioactivity - CPM Incubation Periods			Cells/test	180 min. period ($\times 10^{-4}$)
		45 min.	90 min.	180 min.		
New Brunswick, N. J.	1 AB	18,138	22,341	17,936	1.3×10^8	1
	1 C	31,891	50,297	63,862	1.1×10^8	6
	1 F	7,981	5,396	5,032	4.2×10^6	12
	1 E	16,015	28,892	46,336	5.0×10^5	927
Rocky Mt., Colo.	2 AB	1,318	757	1,250	1.2×10^7	1
	2 D	58,555	61,045	62,288	5.2×10^4 ⊕	11,978 ⊕
	2 G	18,957	26,626	31,158	5.1×10^6	58
Apple Valley, Calif.	3 A***	77	101	143	2.4×10^5	6
	3 B	15,176	21,131	65,848	1.4×10^7	46
	3 C	2,795	4,023	12,287	1.6×10^5	777
	3 G	5,560	11,498	37,864	3.6×10^4	1052
Iron-rich, Orange, Va.	5 OP	12,742	15,466	36,124	6.6×10^4	5470
	5 U	17,539	23,055	43,340	7.0×10^5	619

* M8 plus C^{14} Substrates

	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL-sodium lactate-1	5.0	0.26	1.3	0.002

** Medium - 0.5 ml, M8
Inoculum - 0.1 ml isolate
100 mg. sterile soil

*** A streptomycete. All other isolates are bacteria.

⊕ Being rechecked.

C. ANAEROBIC DETERMINATIONS

Positive evidence of oxygen on Mars has not been found. If it is present, the concentration is very low. The Gulliver probe, therefore, must be able to detect anaerobic metabolism. When the usual methods of producing anaerobiosis in the laboratory were adapted to radioisotope techniques, only the use of the Brewer Anaerobic Jars was successful. Anaerobic soil microorganisms were detected with this method from inocula taken directly out of the Gulliver instrument following a field trial (Section IIA); positive anaerobic photosynthetic responses were also obtained in the Brewer Jar from R. rubrum. This method, however, proved to be quite limiting. A period of approximately 45 minutes was required to obtain complete anaerobiosis; this in turn, reduced the initial obtainable response. Due to the configuration of the Jar, geiger tubes could not be inserted for continuously monitoring the $C^{14}O_2$, not only restricting the gas collection to wet $Ba(OH)_2$ collection planchets, but also limiting the number of collections that could be made.

To overcome these difficulties an anaerobic cabinet was designed by our instrumentation section, and installed in the laboratory. This unit, pictured in Figures III-9A and B consists of a rectangular metal box (12 and 1/2 inches high, 31 inches wide, 4 inches deep) with a removable front panel. Two interchangeable panels were made. A metal one has 12 outlets for external inoculation, each in juxtaposition with a culturing chamber. The other is a plexiglas panel which permits the entrance of light for photosynthetic determinations. A maximum of 12 mock up Gulliver units with their accompanying geiger tubes may be placed within the cabinet for simultaneous, automatically recorded determinations. The leads to the geiger tubes form a common cable which exits through a sealed electrical outlet. Two gas control valves are attached to one

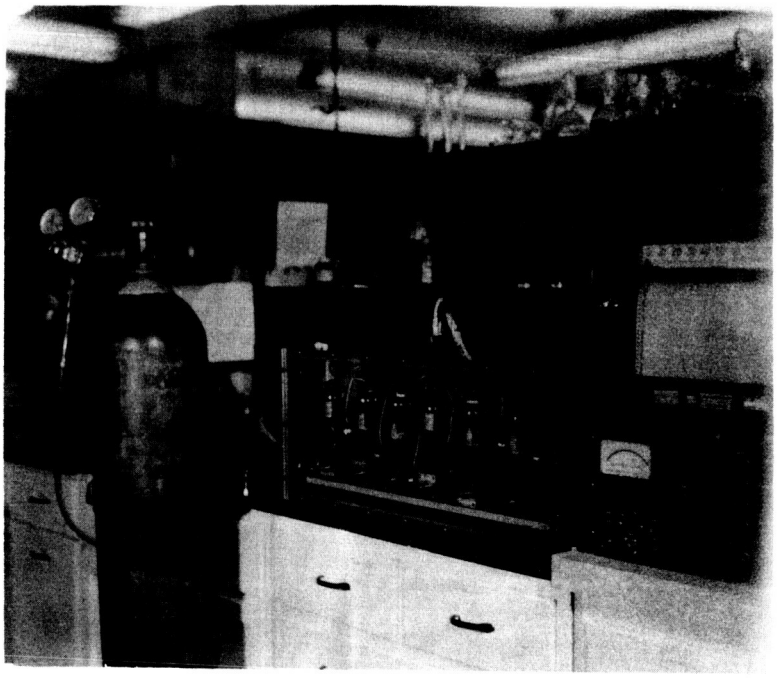
Figure III-9A

ANAEROBIC CABINET

Metal Front Plate for External Inoculation

Figure III-9B

ANAEROBIC CABINET



Plexiglas Front Plate for Photosynthetic Experiments

side of the cabinet. The inlet valve leads to the regulator of a tank of prepurified (99.997% pure) nitrogen; the outlet valve is connected during the flushing operation to a vacuum pump and during sustained operation, a liquid anaerobic indicator and bubble rate meter are inserted in the train.

The general procedure for establishing anaerobiosis follows:

1. Gulliver chambers containing radioactive media are positioned under geiger tubes having $Ba(OH)_2$ Krylon coated aluminized mylar $C^{14}O_2$ collectors over the windows.

2. The front panel is secured in place.

3. Positive N_2 pressure of 1 and one/half inches of mercury is applied. All valves are closed for two minutes and the pressure level checked for any indication of leakage.

4. Flushing of the cabinet follows. The positive pressure of 1 and one/half inches of mercury is alternated with a negative pressure of 1 and one/half inches (evacuation being obtained by means of a vacuum pump). The cycle is repeated five times if chambers only are in the cabinet, ten times if anaerobic plates are included.

5. Once anaerobiosis has been established (approximately 20 minutes is presently required), one of two methods may be used to sustain the condition.

a. The presence of positive pressure alone has been sufficient. This is obtained by closing the outlet valve and adjusting the gas regulator until 1 and one/half inches of pressure is obtained.

b. If a continuous flow of nitrogen is desired, the outlet and inlet valves are adjusted to obtain the 1 and one/half inches of positive pressure. The rate of flow (approximately 50/minute) can be gauged by the bubble rate meter connected to the outlet tubing.

6. The anaerobic inoculum is placed into a syringe and is injected through the front panel ports directly into the culture chamber. The positive pressure is decreased slightly, but not removed, at this time to facilitate injection.

7. Except for periodic checks of the indicators (described below) and pressure level, the system maintains itself; the radioactivity is monitored by the log rate meter and automatically recorded.

An internal and/or external indicator are included to check the anaerobic state. The one within the cabinet can be observed continuously through the plexiglas panel; if the metal panel is in place, the indicator is checked at the completion of the test. An external closed system liquid indicator is connected to the outlet valve when a continuous flow of nitrogen is used; if positive pressure is maintained in the absence of flow, the outlet valve is closed, negating its use. This extremely sensitive indicator* depends on the change of methylene blue from the colored (oxidized) to the colorless (reduced) state.

The anaerobic cabinet has proved to be a very useful laboratory addition. Numerous trial runs were performed to establish the procedure and occasional modifications continue to be made when indicated. The Mark II mock up chambers were used in the first set of experiments. The chambers, each containing 0.5 ml of C^{14} medium and 0.1 ml of inoculum, were positioned in the cabinet, placed under anaerobic conditions, and monitored for 20 to 25 hours. Positive pressure was used to maintain the anaerobic state. The second set of experiments made use of the

* Fildes procedure: Equal parts of N/10 NaOH, methylene blue, and dextrose solutions are boiled until decolorized and immediately placed in the system requiring the indicator.

Mark III mock up chambers. The chambers, containing only the C^{14} medium, 1.0 ml (no inoculum) were placed in the cabinet; after anaerobiosis was established, 0.2 to 0.8 ml of anaerobic inoculum was introduced into the individual chambers from the outside of the cabinet through the ports. In this fashion, the organisms were never exposed to aerobic conditions. A constant flow of nitrogen was used to sustain anaerobiosis. This is the procedure currently used.

Labeled AC medium was used in all determinations. This medium, a modification of Difco's AC medium for aerobic as well as anaerobic microorganisms, forms a large part of the basal M5 medium (Table III-1). The ingredients are listed below:

Proteose peptone #3	20 g
Beef extract	3
Yeast extract	3
Malt extract	3
Ascorbic acid	0.2
per liter	

C^{14} substrates	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.24	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL-sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.4	0.22	1.0	0.002

The M9 and other developmental media are presently being used in comparative determinations. Three anaerobic cultures have been tested to date: Clostridium sporogenes, Clostridium perfringens, and Clostridium pasteurianum. Additional organisms have been ordered for the collection.

Plate counts, performed in all determinations, yielded inconclusive results. Fisher's Brewer Anaerobic Agar was used with Brewer anaerobic dishes, and also with regular petri dishes placed within the anaerobic cabinet for incubation. Either inconsistent colony counts resulted, or stormy fermentation rendered counting impossible. Studies

are under way exploring various methods that might yield reliable cell number determinations of the anaerobic organisms.

The radioisotope response obtained from eleven determinations were very encouraging. Until cell numbers are available, quantitative evaluation cannot be made. However, metabolism was detected in all three organisms, and, in some determinations, exponential growth resulted. (Figure III-10).

As an additional precaution, the purity of the cultures producing positive anaerobic responses was checked. Immediately following the opening of the cabinet samples were taken directly from the incubating chambers, Gram stained, inoculated on nutrient agar slants and incubated aerobically. In all instances, only the desired organisms appeared.

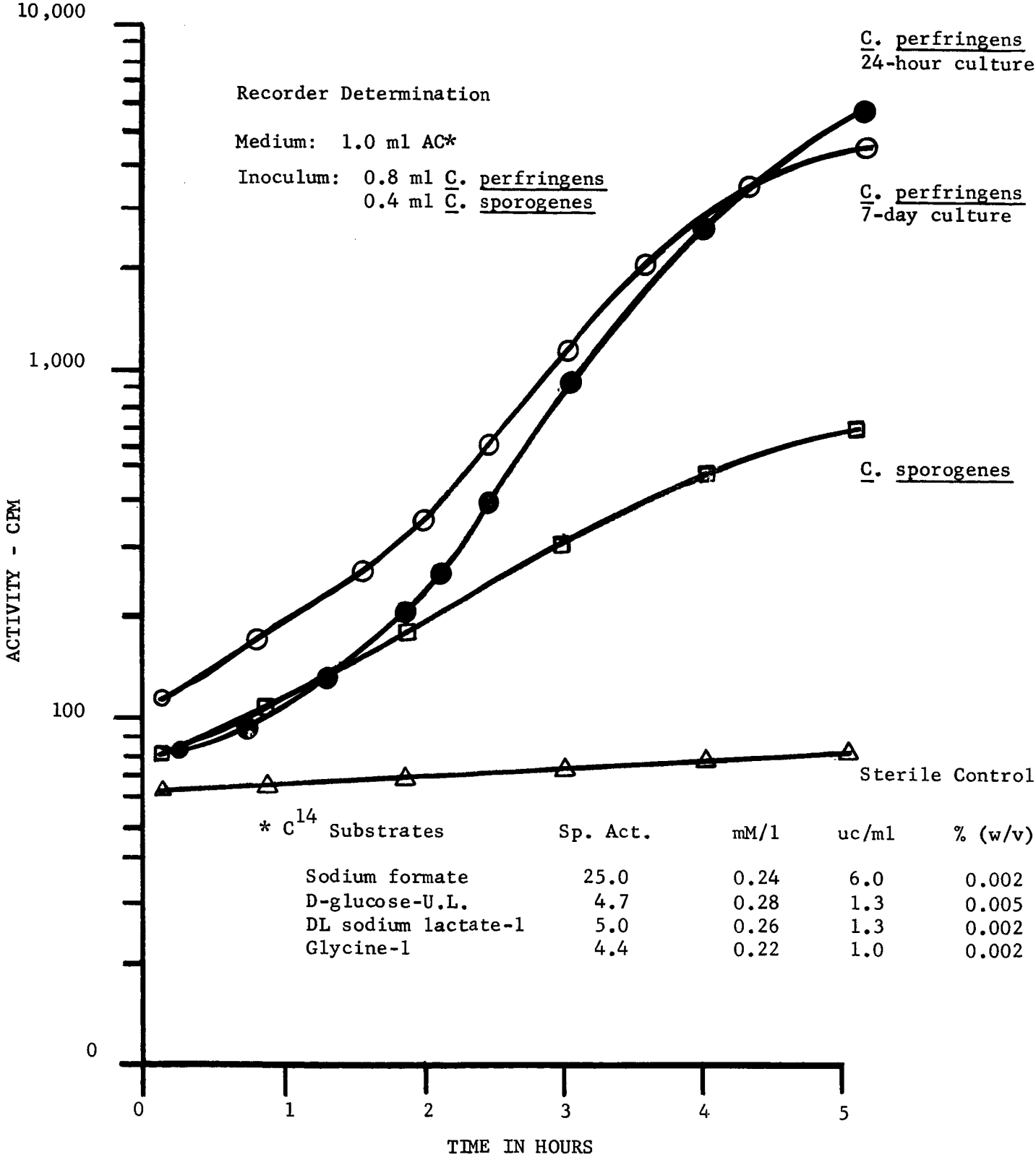
The earliest positive growth response (twice the sterile control level) was obtained in one hour from a seven day culture of C. perfringens, Figure III-10. The medium, in a Mark III unit, was seeded anaerobically after an anaerobic atmosphere was created in the chamber. A 24 hour culture of the same organism responded positively in two hours under the same conditions. Mechanical difficulties forced early termination of the test; however, the five hour period was a sufficient length of time to detect definite growth. Figure III-10 illustrates the growth curves. The difference in the age of the two cultures and their resultant responses provided interesting data. Responses obtained from heterogenous populations of soils are from organisms in many varied stages of growth. The ability of the system to detect metabolism from a week old culture as well as from an actively metabolizing 24 hour culture was illustrated by this determination.

The detection of anaerobic metabolism will continue to be a major part of the program. Soils and pure cultures of facultative and

Figure III-10

ANAEROBIC CABINET DETERMINATION

RESPONSE OF CLOSTRIDIUM PERFRINGENS AND CLOSTRIDIUM SPOROGENES



* C^{14} Substrates	Sp. Act.	mM/1	uc/ml	% (w/v)
Sodium formate	25.0	0.24	6.0	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.4	0.22	1.0	0.002

strict anaerobic microorganisms will be used as inocula. Anaerobic-photosynthetic determinations will be performed and the effects of the various basal C^{14} media will be investigated.

D. ANTIMETABOLITES

Laboratory studies have continued in an effort to select a compound suitable for inhibiting metabolism in the Gulliver control unit. During the past year, Bard Parker, a commercial germicide (isopropanol - 65%, methanol - 3%, formaldehyde - 8%, and hexachlorophene - 0.5%) has been used successfully in the laboratory and field. However, additional compounds were screened to assure the selection of the most effective inhibitor. In addition to the prime prerequisite of possessing a wide antimicrobial spectrum against soil microorganisms, the antimetabolite must be able to withstand sterilization temperatures of 135°C for 26 hours, be chemically unreactive with the labeled medium constituents to avert production of nonmetabolic $C^{14}O_2$, and readily able to penetrate to the inoculum in the chamber.

Compounds tested were acrolein (CH_2=CHCHO), Argyrol (a commercial preparation of 10% silver protein) and mercuric chloride ($HgCl_2$). Experimental details of the determination follow:

Method ----- Planchet

Medium ----- M8, 0.5 ml plus 0.1 ml of inhibitor

C^{14} Substrates -----	Sp. Act. (mc/mM)	mM/l	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	4.7	0.28	1.3	0.005
DL-sodium lactate-1	5.0	0.26	1.3	0.002

Inocula ----- Iron-rich soil (Orange, Va.), 100 mg

E. coli, 0.1 ml of a diluted broth culture

Successive incubation periods ----- 2.0 and 4.0 hours

$C^{14}O_2$ collection periods ----- 15 minutes following each incubation period

Inhibitors ----- Final concentration

Acrolein	0.002%, 0.003%
Argyrol	1.0%
Mercuric chloride	0.2%

Results ----- Acrolein - high control values, no inhibition in soil, partial inhibition of *E. coli*

Argyrol - high, inconsistent control values, partial inhibition

Mercuric chloride - chemically reactive with medium

None of the compounds tested were suitable.

The Bard Parker germicide, used extensively in the field tests, continues to be the most effective inhibitor. In each field trial two identical instruments were operated simultaneously. Following inoculation of both chambers, one instrument was allowed to operate uninhibited, the other received the antimetabolite, either immediately or shortly after metabolism was detected. The effect of the inhibitor upon the metabolism or growth of the soil microorganisms was definitely apparent. Figures II - 8, 9 depict results obtained with immediate injection of Bard Parker. Figures II - 2, 3, 6, 7, 11, 12, 13, 14, 15 are the result of delayed antimetabolite injection. In all instances the inhibitor functioned satisfactorily.

Investigation of chemical antimetabolites will continue. The Bard Parker will be used until a more effective inhibitor is found. If a more effective inhibitor is found and it is compatible with experimental requirements, it will be used.

E. PHOTOSYNTHESIS

The ability of "Gulliver" to detect life has been amply illustrated, rather spectacularly, in the extreme environmental field tests. The metabolism detected has been primarily from non-photosynthetic organisms. If Mars is inhabited by microbial life, the strong possibility exists that photosynthesis plays an important role in all or many of the organisms. If this is so, it is most desirable for the Gulliver instrument to detect and distinguish both forms of metabolism.

It was shown (Second Annual Progress Report) that the photosynthetic alga, Chlorella pyrenoidosa, could heterotrophically assimilate a labeled medium, and produce $C^{14}O_2$. This $C^{14}O_2$ could subsequently be fixed photosynthetically. Measurable responses indicated differences in net $C^{14}O_2$ evolved under light and dark culturing conditions. The program was continued and further expanded this year.

The laboratory procedures described fully in the Second Annual Progress Report will be briefly reviewed here. The Gulliver mock up chambers were modified slightly, permitting light to enter the incubation chambers. This was accomplished by (1) replacing one side of each chamber with a sliding panel (2) inserting a small pyrex test tube to serve as the actual incubation chamber and (3) making a gas tight seal at the top of the tube. Labeled medium and inoculum placed in the incubation tube-chamber can be alternately exposed to light or dark through use of the sliding panel. The evolved $C^{14}O_2$ can be collected and monitored by means of the automated monitoring and recording unit and also by wet $Ba(OH)_2$ planchet collections. If the latter method is used, a sliding panel is placed over the open top of the incubation chamber. During the gas collection periods the panel is moved to the open position.

The experimental procedure is as follows: labeled media is introduced into three sets of chambers and inoculated with C. pyrenoidosa. One set is initially exposed to light; the second set is initially maintained in the dark; the third set forms the controls, one of which is kept continuously in the dark; the other continuously in light. The cultures are allowed to incubate for a prescribed time period at room temperature after which $C^{14}O_2$ collections are made with planchets or assayed by the Geiger Müller tubes in the automatic recording unit. The chambers initially receiving light are then put into the dark phase by closing the sliding panels. Those initially in the dark are exposed to the light. Following the second incubation period $C^{14}O_2$ is again assayed. This cycle is repeated several times.

Determinations performed during this year measured (1) the effect of several light-dark cycles (2) the effect of increased labeled substrate concentration (3) the importance of initial light exposure versus initial dark exposure (4) the responses to basal medium containing KNO_3 in place of urea and (5) a preliminary study using Scenedesmus quadricauda as inocula.

Results of previous investigations indicated that DL-sodium lactate-1-C^{14} was a good radioactive substrate to incorporate into the basal medium. Experiments were performed to determine the effects of the lactate-C^{14} concentration upon the responses. Concentrations of 1×10^{-3} M, 5 uc/ml and 2×10^{-3} M, 10 uc/ml of DL-sodium lactate-1-C^{14} were added to basal urea media. One half ml of each medium was inoculated with approximately 5×10^6 algal cells. Five light-dark cycles were run, and the evolved $C^{14}O_2$ collected with planchets. The cultures (Figure III-11 and III-12) exhibited a more marked response to light change with the higher concentration of lactate. It was also observed that by starting

the inoculated chambers in the light, the initial response to light change was more pronounced. The higher molar and radioactive concentration, 2 x 10^{-3} M and 10 uc/ml of lactate-C^{14} was incorporated into the basal medium.

Throughout the algal study a basal salt medium with urea as a nitrogen source has been used. The medium constituents are:

	Urea	1000 mg
	$MgSO_4 \cdot 7H_2O$	1000
	KH_2PO_4	250
Sequestrene	NaFe	35
" "	Na_2Mn	3
" "	Na_2Zn	1
" "	Na_2Co	1
" "	Na_2Cu	1
	(agar 1%)	
	water to one liter	

The use of KNO_3 as a nitrogen source was investigated. The urea in the basal salts medium was replaced with 3.42 g/l of KNO_3. C. pyrenoidosa, cultured in the KNO_3 salts medium, was used as the inoculum with KNO_3 salt medium containing 2 x 10^{-3} M of lactate-C^{14} in a light-dark chamber experiment. The responses obtained to light change were the same as those with the urea-salts medium. The absolute levels of evolved $C^{14}O_2$ however, were lower with the KNO_3, due perhaps to the high pH, 8.5, of the KNO_3 medium in comparison to the 6.5 pH of the urea medium. Since the KNO_3 did not appear to be of added benefit, the urea medium was selected for normal use.

Studies are continuing on different genera of algae. The results will be presented at a later date.

A new self-contained photosynthetic chamber, Figure III-13 has been designed by our Instrumentation Section. One unit has been

Figure III-11

$C^{14}O_2$ EVOLVED BY CHLORELLA PYRENOIDOSA
IN RESPONSE TO LIGHT AND DARK GROWTH CYCLES

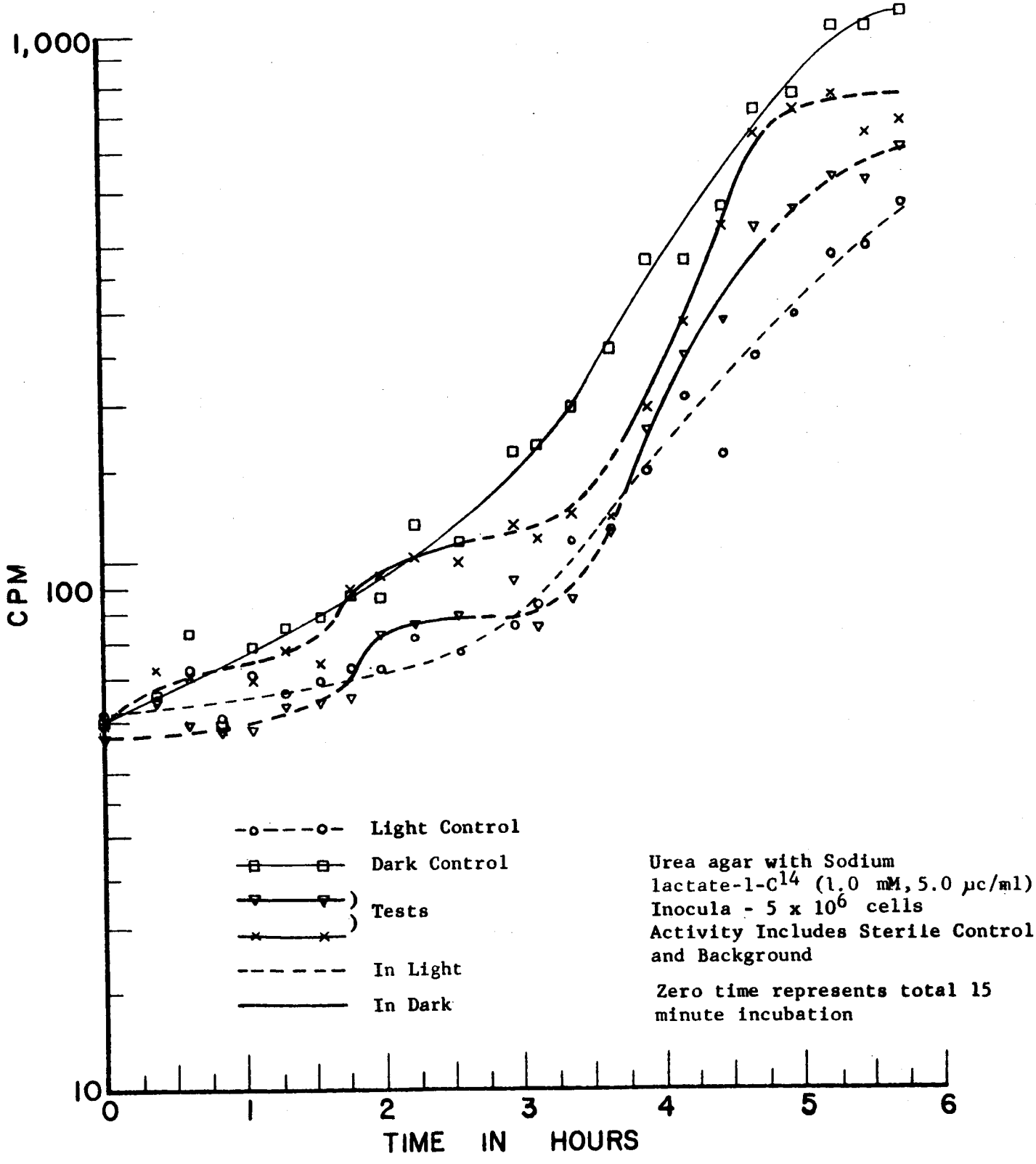
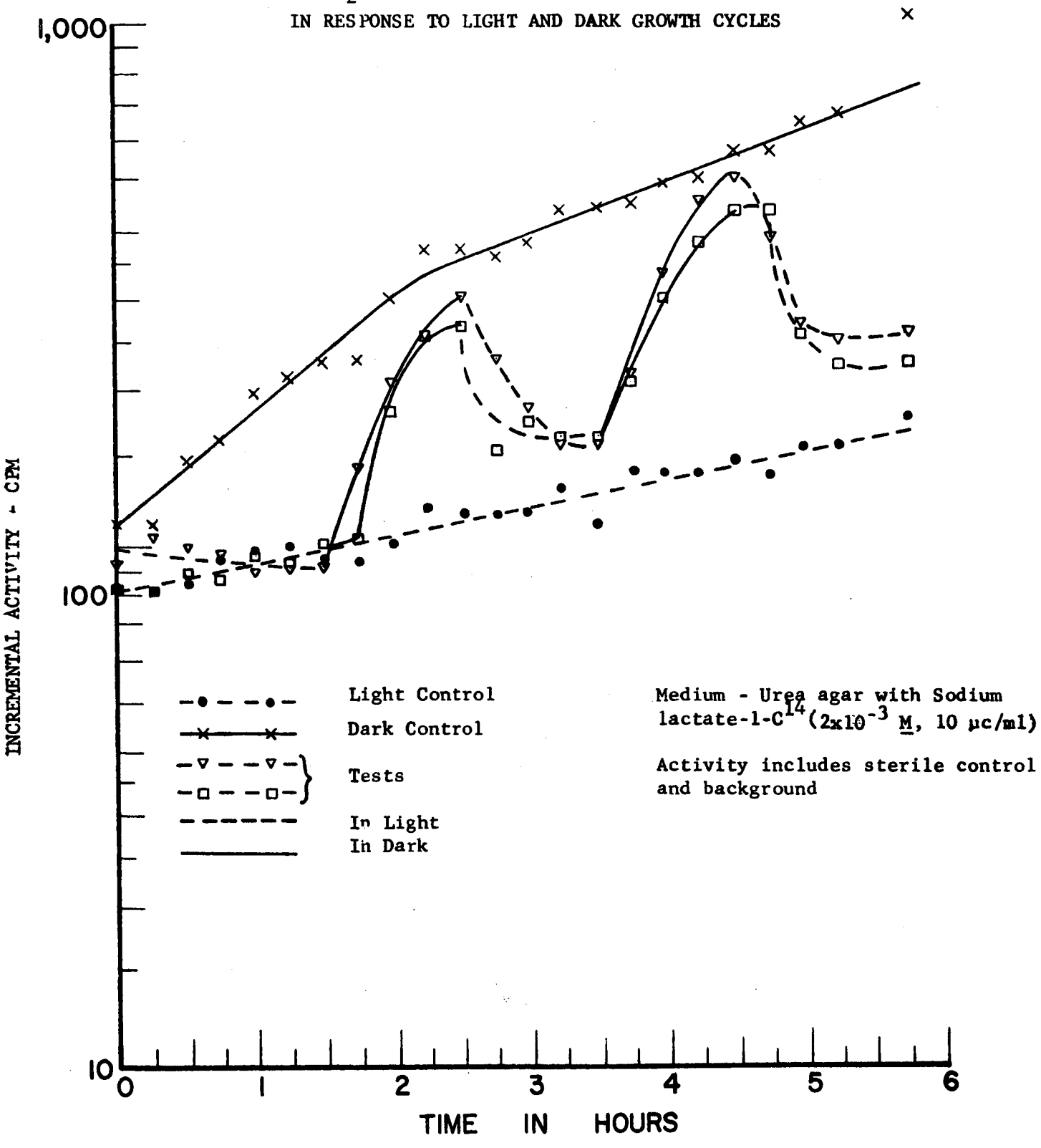


FIGURE III-12

$C^{14}O_2$ EVOLVED BY CHLORELLA PYRENOIDOSA
IN RESPONSE TO LIGHT AND DARK GROWTH CYCLES

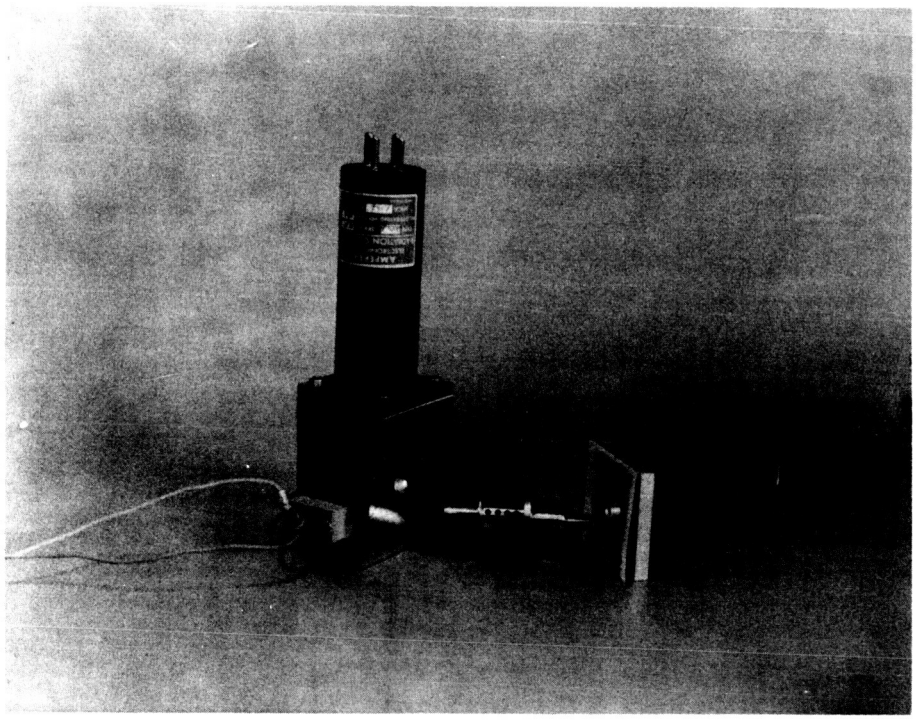


built by modifying a Mark III mock up chamber. Two Chicago Minature Light Bulbs #334 were placed at 180^{o} angles inside the actual culturing chamber. The two externally placed adaptors which position the bulbs are connected to a rheostat which permits selection of light intensities. The unit, recently compiled, is presently undergoing testing.

A new approach, more suitable for an experiment on Mars is now being studied. A possible difficulty with the present photosynthesis experiment is that it relies upon a heterotrophic capability in the organism. A strict autotroph, as might be encountered in the actual experiment, would not incorporate any of the labeled organic substrates used. It would be better if the experiment were freed of this constraint. An attempt is being made to accomplish this in the following manner. The photosynthetic organisms will be exposed to $C^{14}O_2$, HCO_3^- or CO_3= or any combination thereof, and light. Photosynthetic activity will incorporate the C^{14} into starch and other products of carbohydrate metabolism. Any gaseous $C^{14}O_2$ will then be removed from the culture chamber and the light will be excluded. The organisms will now have to resort to endogenous sources of energy stored up during photosynthesis. This material now contains C^{14} which should be expired as $C^{14}O_2$ and detectable by the standard Gulliver procedure.

Figure III-13

MARK III PHOTOSYNTHETIC CHAMBER



F. MANAGEMENT, PERSONNEL, CONFERENCES, PUBLICATIONS

Management

Resources Research, Inc., Washington, D. C. became a wholly owned subsidiary of Hazleton Laboratories, Inc., Falls Church, Virginia. The space-biology section is now part of a newly created Bioengineering Department of Hazleton Laboratories.

Personnel

Dr. John M. Barnes, plant pathologist, and Duane G. Hoffman, design engineer, have become associated with the Radioisotopic Biochemical Probe for Extraterrestrial Life. Dr. Barnes will be concerned with the photosynthetic aspects of the program. Mr. Hoffman has assumed responsibility for quality control procedures, minor instrument modifications and mechanical and electronic maintenance.

Conferences

On June 26, 27, 1963, Dr. L. Hockstein and Mr. J. Cole of the Ames Research Center, Palo Alto, California, appraised the Gulliver experiment as part of an overall NASA review of space probe experiments. A field demonstration of the instrument was included (Figure II-7). Personnel from Resources Research, Incorporated, American Machine and Foundry Company, and the Ames Research Center were in attendance.

On July 10-12, 1963, conferences were held with personnel from Resources Research, Incorporated; American Machine and Foundry Company; Jet Propulsion Laboratory; and Dr. Norman Horowitz, California Institute of Technology, at the Jet Propulsion Laboratory, Pasadena, California.

Dr. G. V. Levin participated in the Space Biology Workshop, held at the University of Rochester, Rochester, New York, on January 9, 10, 1964.

Publications

(1) "GULLIVER" An Experiment for Extraterrestrial Life Detection and Analysis was presented June 10, 1963 at the COSPAR Fourth International Space Science Symposium, Warsaw, Poland.

(2) The Design of Martian Biological Experiments presented June 10, 1963 at the COSPAR Fourth International Space Science Symposium, Warsaw, Poland.

(3) Rapid Microbiological Determinations with Radioisotopes, Advances in Applied Microbiology; Vol. 5 (1963).

Acknowledgements

The investigators wish to thank Mr. J. B. Carter, Mr. C. J. Koch, and the Virginia Polytechnic Institute for all their assistance and for providing a site for testing purposes.

They would also like to acknowledge the participation and generous aid of Dr. Gerald Soffen and Dr. Roy E. Cameron of the Jet Propulsion Laboratory, Pasadena, California. Their knowledge of the California area, provision of vehicles and equipment, and assistance during the testing were invaluable. Their atmospheric measurements at the field sites and analysis of the field test soils, presented in Tables II-2A, B, C provided valuable information, particularly concerning the nature of the soils tested. Credit is also due to Dr. Soffen for his excellent photographic coverage of the field trials, illustrated by the fine Gulliver plates throughout the report.

In addition, grateful acknowledgement is given to the Biology Division of the California Institute of Technology for furnishing equipment and laboratory facilities and to the Crooked Creek Laboratory of the University of California for use of their facilities during the

California field trials.

The investigators would also like to express their appreciation to the following people for providing the test collection with special cultures: Dr. E. G. Mulder, Agricultural University, Wageningen, The Netherlands, who sent strains of Sphaerotilus and Leptothrix organisms; Dr. H. L. Ehrlich, Rensselaer Polytechnic Institute, Troy, New York, for Ferrobacillus ferrooxidans; and Mrs. B. H. Caminita, U.S. Naval Weapons Laboratory, Dahlgren, Virginia, for Serratia marcescens and B. subtilis var globigii.

G. TECHNIQUES, MISCELLANEOUS

(1) Techniques

Planchet Determination

1. Two planchets are required per test; one for incubation, one for gas collection.
2. The sterile incubation planchet is contained in a sterile petri dish.
3. Labeled medium and inoculum are placed and gently mixed by swirling in the incubation planchet.
4. The lid of the petri dish is replaced and the culture is allowed to incubate at a desired temperature for a selected time period.
5. The gas collector is prepared by placing approximately two drops of saturated $Ba(OH)_2$ solution on a filter paper pad placed securely in the collection planchet.
6. The labeled gas evolved from the culture is collected for a selected time period (usually 15 minutes) by inverting the prepared collecting planchet over the incubation planchet.

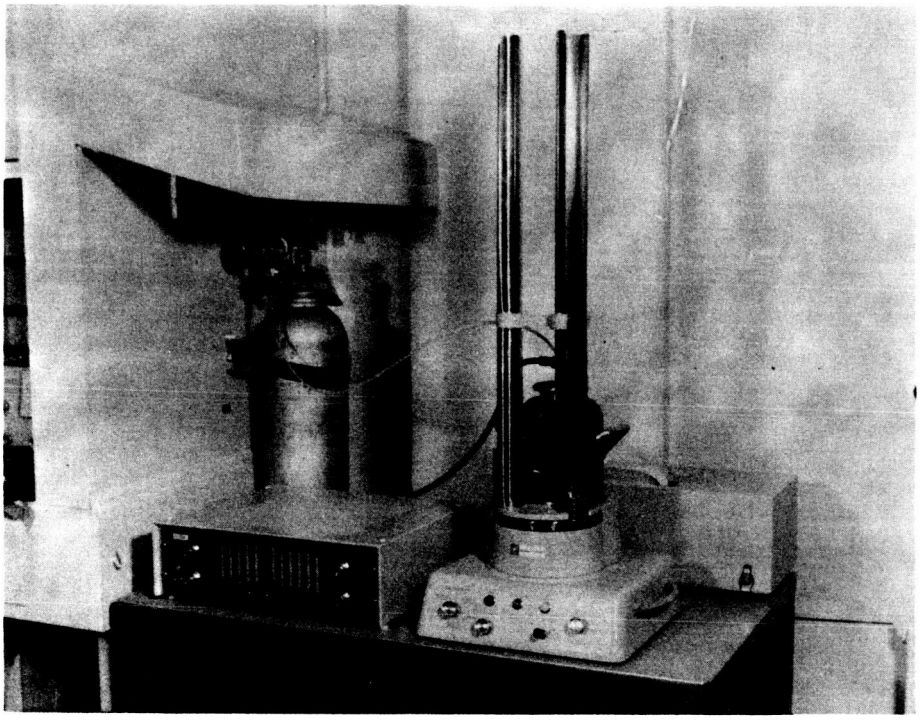
7. The collecting planchet is removed and placed under an infrared lamp until dry.
8. The radioactivity on the pad's surface is counted in the D-47 gas flow counter pictured in Figure III-14.
9. If successive responses are desired, incubation of the cultures is continued and another collection is made. The cycle is repeated as often as needed.

Automatic Recorder Determination

1. One sterile mock up Gulliver Mark III chamber is required per test.
2. A gas collector is prepared by mixing $Ba(OH)_2$ powder and Krylon. This mixture is quickly and evenly painted onto the aluminum side of aluminized mylar.
3. Labeled medium is placed into the incubation section of the chamber.
4. The medium is inoculated with the desired microorganism or soil.
5. Baffles, to prevent direct reading, are put into place.
6. The prepared collector is fitted over the chamber top opening, with the $Ba(OH)_2$·Krylon side facing the culture chamber, and secured by an O ring.
7. The entire chamber is placed under a Geiger Müller tube. The tube fits securely into the top opening of the chamber, and faces the non-collecting side of the mylar collector.
8. The responses are then automatically and continuously recorded by the log rate meter and recorder for a selected time period.
9. Twelve determinations may be made simultaneously.

Figure III-14

RADIOACTIVE COUNTING EQUIPMENT



The D-47 gas flow counter is to the right of the scaler; the printer-timer appears behind the counter.

Anaerobic Determination

The anaerobic cabinet is now used for all determinations. The procedure is fully described in Section IIIC.

(2) Culture Media

The stock cultures are maintained and prepared for use with Nutrient Agar and Nutrient Broth, unless specific culturing media are required. The composition of the various media are presented below.

NUTRIENT BROTH (Difco, Fisher)

Beef Extract, Bacteriological	3.0 g
Peptone	5.0
pH 6.9	per liter

NUTRIENT AGAR (Difco, Fisher)

Peptone	5.0 g
Beef Extract, Bacteriological	3.0
Agar	15.0
pH 6.8	per liter

SABOURAUD LIQUID MEDIUM (Fisher) Yeasts, Molds, Fungi

Pancreatic digest of casein	5.0 g
Peptic digest of animal tissue	5.0
Glucose	20.0
pH 5.7	per liter

SABOURAUD DEXTROSE AGAR (Fisher, Difco) Yeast, Molds, Fungi

Multi-Peptone (Fisher) (neopeptone-Difco)	10.0 g
Dextrose	40.0
Agar	15.0
pH 5.6	per liter

BREWER ANAEROBIC AGAR (Fisher) Anaerobes

Tryptone	17.5 g
Soy peptone b.	2.5
Sodium Chloride	2.5
L-Cystine	0.4
Glucose	10.0
Agar	15.0
Sodium thioglycollate	2.5
Sodium formaldehyde sulfoxylate	1.0
Methylene blue, certified	0.002

pH 7.2 per liter

FLUID THIOGLYCOLLATE MEDIUM (Fisher) Anaerobes

Pancreatic digest of casein, U.S.P.	15.0 g
Yeast extract, bacteriological	5.0
L-Cystine	0.5
Glucose	5.5
Sodium chloride	2.5
Sodium thioglycollate	0.5
Resazurin	0.001
Agar	0.75

pH 7.1 per liter

COOKED MEAT MEDIUM (Fisher) Anaerobes

Beef heart	454.0 g
Proteose peptone	20.0
Dextrose	2.0
Sodium chloride	5.0

pH 7.2 per liter

TRYPTONE GLUCOSE EXTRACT AGAR (Difco, Fisher)

Tryptone	5.0 g
Beef extract, bacteriological	3.0
D-glucose (Dextrose)	1.0
Agar	15.0

pH 7.0 per liter

TRYPTONE GLUCOSE EXTRACT BROTH (Fisher)

Tryptone	5.0 g
Beef extract, bacteriological	3.0
D-glucose	1.0

pH 7.0 per liter

LACTOBACILLUS AGAR (Fisher) L. plantarum

Lactalbutone	20.0 g
Sodium chloride	2.0
Sodium citrate	3.0
Glucose	9.5
Agar	13.5

pH 7.0 per liter

GREEN-TOP MEDIUM R. rubrum

Yeast extract	0.2%
Bacto-tryptone	0.1%
Sodium acetate	0.1%
Soil extract	0.2%
(Agar)	(1.5%)

pH 7.2

THIOBACILLI MEDIUM T. thiooxidans, T. novellus

$(NH)_2SO_4$	0.2 g
$MgSO_4$	0.5

THIOBACILLI MEDIUM (Cont'd) *T. thiooxidans*, *T. novellus*

$CaCl_2$	0.25
$FeSO_4$	trace
KH_2PO_4	3.0
precipitated sulfur	10.0
	per liter

Mn AGAR Iron bacteria

$MnCO_3$	2.0 g
Beef extract	1.0 g
Agar	7.5 g
Ferrous ammonium sulfate	150.0 mg
Yeast extract	75.0 mg
Sodium citrate	150.0 mg
Cyanocobalamin	0.005 mg

Soil Extract

500 g of air-dried soil are placed in 1300 ml of water containing 0.1% Na_2CO_3. The mixture is autoclaved one hour, filtered and made up to 1000 ml with water.

Third Annual Progress Report

NASr-10

Respectfully submitted,

Gilbert V. Levin

Gilbert V. Levin, Ph.D.
Experimenter

Norman H Horowitz

Norman H. Horowitz, Ph.D.
Experimenter

Allen H. Heim

Allen H. Heim, Ph.D.
Senior Microbiologist

Mary-Frances Thompson

Mary-Frances Thompson
Biologist

SECTION IV

INSTRUMENTATION

A. Wendell Carriker
Project Manager

Dr. A. A. Sterk, Manager
Space Instrumentation Dept.

AMERICAN MACHINE & FOUNDRY COMPANY
RESEARCH & DEVELOPMENT DIVISION
1025 NORTH ROYAL STREET
ALEXANDRIA, VIRGINIA

IV. INSTRUMENTATION

PART I. REPORT ON LAST QUARTER

A. SUMMARY

During this last quarter the efforts were focused almost entirely on evaluation of parameters related to the efficiency of radioisotope detection. The Gulliver III instrument employs a single geiger tube with an 0.87 inch diameter end window about 1.7 mg/cm^2 thick for detection of the C^{14} beta particles from the gas collector which is mounted on the window. There have been experimental results which indicated that sensitivity for a given quantity of $C^{14}O_2$ gas could be improved if a detector could be employed that did not require a window or had a larger area thin window. In the last few quarterly reports the problems associated with use of a windowless counter were discussed. The most recent report discussed tests that were conducted with a windowless counter system that would not require exposure of the microbial culture to the special gases required for operating the detector, and in which the culture would always be in the gases of the natural environment. The increase in sensitivity of that detector system over the detector system presently in Gulliver III appeared to be about a factor of five--a figure that is not very attractive when consideration is given to the penalty that would be paid in size, weight, power, and complexity for a flight instrument.

Tests were conducted this last quarter to determine the effect of detector window area on sensitivity to a given quantity of $C^{14}O_2$ gas. The results indicated that under certain conditions the area of the detector window has little effect on the system sensitivity if the quantity of $C^{14}O_2$ is much less than the capacity of the gas collector used. If a gas collector of a given thickness (mg cm^{-2}) and area capacity (moles of CO_2 collected per unit collector area) is to be used, it appears that increasing the area of the detector window permits increasing collector capacity for $C^{14}O_2$. This permits maintaining a better affinity for moderate quantities of CO_2 and permits the instrument to attain higher count rates, in the cases where detector dead-time losses do not limit the count rate.

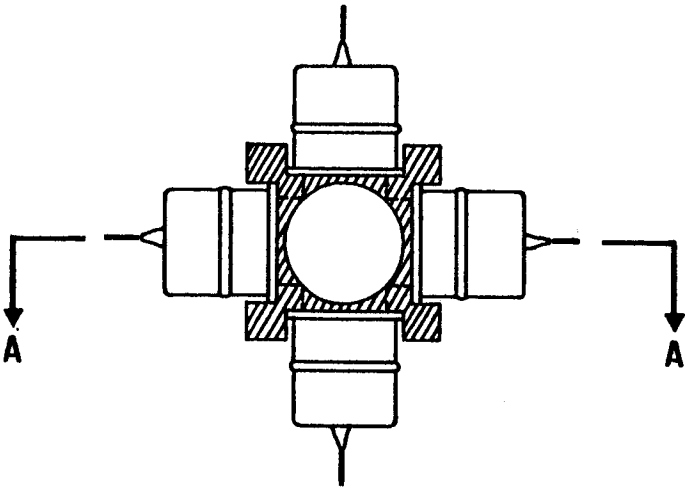
Tests were conducted with a detector that had both a thinner and larger area window to verify the previously mentioned minimal effect of detector window area on sensitivity. This detector operated as a gas flow detector and had a window area that was about a factor of five greater than the geiger used in Gulliver III. This detector showed an increased sensitivity over the thin window geiger, but the causes of increased sensitivity were shown to be related to factors other than window area.

Three special large-area thin window gas flow detectors were obtained and tested in anticipation of use on the Gulliver instruments for a field test. However, the detectors did not operate satisfactorily and were returned to the manufacturer.

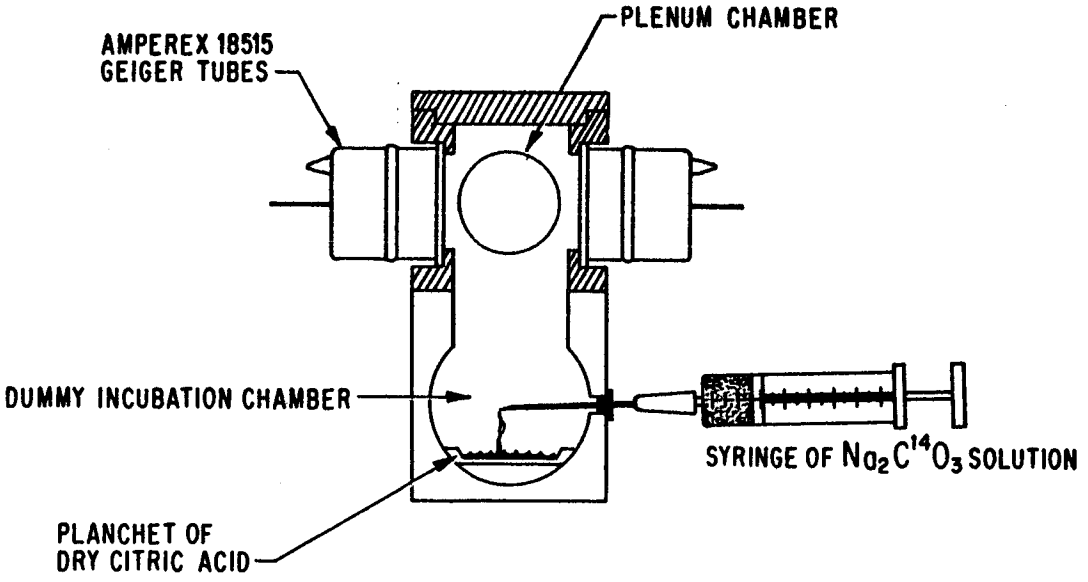
B. TESTS WITH MULTIPLE GEIGERS ON PLENUM ABOVE INCUBATION CHAMBER

There were a number of objectives for this series of tests. These included: (1) Determination of the effect of gas collector, or detector, area on sensitivity; (2) determination of sensitivity attainable with a hollow, thin inner wall, cylindrical detector; (3) determination of the contribution of gas phase CO_2 to the total response of a detector with a gas collector on the window; and (4) determination of the time required for evolved gases to be collected. Results of these tests were then compared to responses obtained with the detector configuration now in Gulliver III.

The apparatus used in the first series of tests is shown in Figure IV-1. This is a dummy incubation chamber of the same interior dimensions as Gulliver III, on top of which is mounted a small plenum chamber with stations for four geiger tubes. $C^{14}O_2$ can be released in the incubation chamber by either metabolic action or by chemical generation. Some of the experiments described here were performed using the latter method since chemical generation furnishes a means of reproducing the quantity of gas released from run to run. The chemical generation of CO_2 was effected by dropping one drop per minute of 1.0 uc/ml, 0.01 m M/ml $Na_2C^{14}O_3$ solution into an aluminum planchet containing powdered citric acid. On the first run, one ml of solution gave thirty-four drops and this number was subsequently used each time. The moles of CO_2 thus liberated (0.01 mM) was always less than the capacity (>0.1 m M) of the LiOH on the collection



SECTION THRU PLENUM CHAMBER



SECTION A-A THRU INCUBATION CHAMBER

Figure IV-1. Apparatus for Tests of CO_2 Detection with Multiple Geigers.

pads.* The conversion of the carbonate to CO_2 was nearly 100 percent since monitoring the acid at the termination of a test indicated little or no residual activity.

The purpose of the gradual chemical evolution, rather than "one shot" generation when all the carbonate is put on the acid crystal at one time, is to simulate metabolic rates. By duplicating the rate each time (i.e., one drop per minute), variations among runs are minimized. This technique provides a basis of comparison of data, with only one significant uncontrolled variable: the collector pad differences. These differences, of course, are also kept to a minimum by using pads of nearly equal weights taken from a given fabrication batch.

For the tests when the CO_2 was generated metabolically, an aliquot sample of a 24-hour E. coli culture was mixed with tagged broth and equal volumes of the mixture were deposited in the two mock-up incubation chambers--one chamber having the plenum chamber with multiple geigers at the top and the other having the single geiger mounted at the top as in Gulliver III. The assumption was made that equal volumes of the mixture would liberate equal volumes of CO_2.

1. Gas Phase Counting and Simulation of Hollow Cylindrical Detector with Thin Inner Wall

In this test four thin window geiger tubes were mounted on the plenum chamber and $C^{14}O_2$ was generated chemically. The count rate from

*Collector pads always had greater than 15 mg of $LiOH \cdot H_2O$ and 1 mg of $LiOH \cdot H_2O$ will convert 0.068 m M CO_2 to Li_2CO_3.

each detector increased steadily as the solution was deposited on the acid crystals. The count rate leveled off at about 10,000 cpm within five minutes after the last drop of solution was deposited. By summing the count rate of all the tubes and normalizing the count rates of each detector by a measured sensitivity factor, a total count rate of about 40,000 cpm is obtained.

When the same quantity of $C^{14}O_2$ was chemically generated in a mock-up unit with the regular Gulliver III detector configuration with a gas collector mounted on the window, the count rate similarly increased steadily until the last drop of solution was deposited and leveled off at about 60,000 to 70,000 cpm within about ten minutes after the last drop--the increase being less than 10,000 cpm during that ten-minute period.

Considering that the windows of the geigers in the plenum chamber cover about 30 percent of the total surface area of the volume of the plenum chamber above the mock-up, a factor of 1/0.3 or 3.33 can be applied to the 40,000 cpm to determine that the count rate would have been about 140,000 cpm with a thin inner wall cylindrical detector. That count rate is based on the further assumption that the ratio of volume of the cylindrical detector to the volume of the incubation chamber would be the same as the volume ratio of plenum chamber to incubation chamber and that, to minimize end losses, the length of the cylindrical detector is large in comparison to its inner diameter.

Comparing the calculated count rate of 140,000 cpm to the average of 65,000 cpm obtained with a single detector with collector in the Gulliver III configuration and looking at geometrical factors, it at first appears that the difference results strictly from geometry because the hollow cylindrical detector has a geometrical efficiency approaching 100 percent while the thin end window has a geometrical efficiency factor of only 50 percent. But, it must be remembered that in the experimental set-up the geigers in the plenum chamber detect only that fraction of the CO_2 which was in the plenum chamber. Much, probably 60 percent, of the CO_2 remained in the incubation chamber since there was no gas collector.

Tests to be described later in the report indicate that little or no CO_2 remains in the gas phase when a collector is used; therefore, in the tests with a single geiger tube with a collector, all the $C^{14}O_2$ was converted to carbonate, and the single geiger monitored the total quantity of C^{14} with a maximum geometrical efficiency factor of 50 percent. Had there been no absorption of the C^{14} betas in the gas collector, probably the count rate would have been about 100,000 cpm. Converting from 50 percent geometry to 100 percent geometry would provide a count rate of 200,000 cpm for the single detector for the quantity of $C^{14}O_2$ generated. By considering that the "volumetric" geometry factor for the plenum chamber was about 40 percent and converting that factor to 100 percent (assuming that the detector volume would be large compared to incubation chamber volume) would yield a calculated count rate of 350,000 cpm for a thin inner wall cylindrical detector

rather than 140,000 cpm. The comparison of theorized maximum count rates of 200,000 cpm for the single geiger with collector to 350,000 cpm for the hollow thin inner wall cylindrical detector is not bad, considering all the assumptions made, and indicates that the measurements taken and assumptions made are reasonably consistent.

At first thought, employment of a hollow, thin inner wall, cylindrical detector would appear to be an attractive possibility. However, no such detectors are available. The development of a space-flight worthy detector of that configuration would entail significant problems. Some of these problems include: supporting the thin window of the inner cylinder wall; minimizing the dead-time problems of a multiple anode detector; maintaining the gas-tight seals or providing a supply of gas and controls for operation in a flow mode; and the other environmental requirements for a space mission.

2. Contribution of Gas Phase $C^{14}O_2$ to Count Rate When a Gas Collector Is Employed, Rate of CO_2 Collection, and Effect of Collector or Effective Detector Area on Sensitivity

For this series of tests four thin-window geiger tubes of the type used in Gulliver III (Amperex 18515) were mounted in the ports of the plenum chamber on top of the dummy incubation chamber. Gas collector pads were mounted on the face of one, then two, and then four of the geigers. In some cases, a baffle was inserted in the plenum chamber to divide the chamber

to prevent geigers on one side of the chamber from detecting activity on collector pads on the other detectors. The $C^{14}O_2$ was generated chemically as in the previous tests.

In the first of these tests, only one of the four geiger tubes had a gas collector pad mounted on the window. The results are shown in Figure IV-2 along with a diagram of the configuration. It is to be noted that the count rate of the geiger tube with the gas collector pad on the window reached only 48,000 cpm, rather than the 60,000 to 70,000 cpm attained in the previous test by the single geiger tube with a gas collector pad when mounted directly on top of the incubation chamber as with the Gulliver III configuration. This discrepancy can easily have resulted from differences in gas collector pads and differences in geiger tube sensitivity. Each of the bare tubes (without gas collector) gave a count rate of about 10 percent of that of the tube with the gas collector. Although the two geigers adjacent to the one with the gas collector are closer, the solid angle they subtend with the collector is about equal to the solid angle subtended by the geiger opposite the gas collector.

The curve showing 10 percent of the count rate of the detector with the gas collector is also shown in Figure IV-2. The difference between values on this curve and the values on the curves of the count rate of each of the bare detectors indicates the contribution of the gas phase CO_2 to the total count rate of the bare tubes. It is evident that shortly after the last

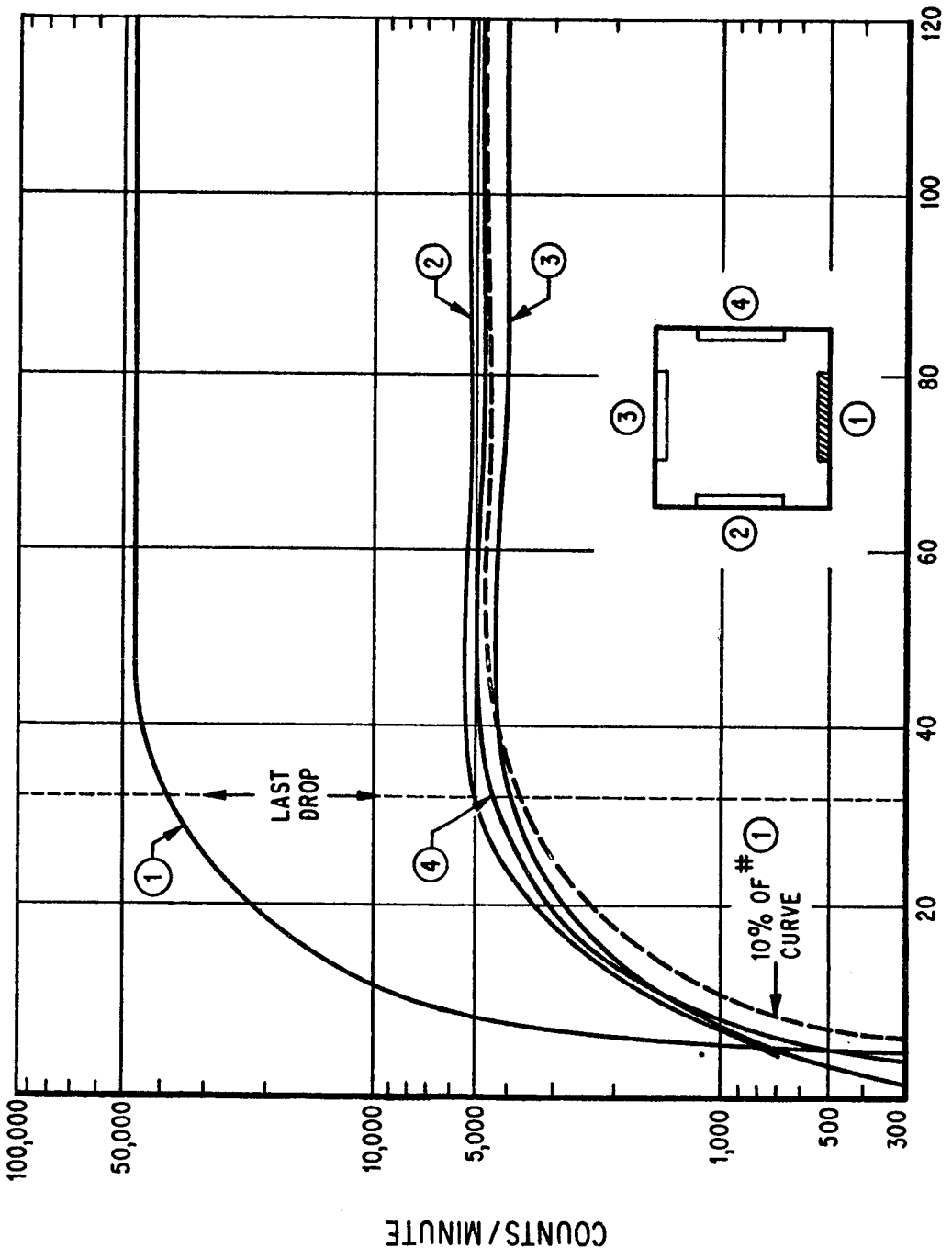


Figure IV-2. Test of One Geiger with Gas Collector and Three Bare Geigers.

bit of CO_2 is generated, none of the detectors detect appreciable gas phase C^{14}. Detectors could be removed from the plenum chamber and then put back and the previous count rate would be observed. This indicated that all the CO_2 had been converted to carbonate by the collector.

In the following test, the small contribution of gas phase $C^{14}O_2$ to total count is even more evident. In this test, the apparatus and procedure was the same as the previous test, except for the baffle which was inserted to divide the plenum chamber. A diagram of this configuration and the results are shown in Figure IV-3. The count rates of the two detectors which were shielded by the baffle from the gas collector pad continued to increase until the generation of CO_2 ceased and then began to decrease with a halving time of 10 to 15 minutes. Since the count rate of the bare tubes is directly related to the quantity of $C^{14}O_2$ in the gase phase, these data indicate that for this set of conditions the time required for half the CO_2 in the chamber to be converted to carbonate is 10 to 15 minutes.

It is noted that the unshielded bare tube adjacent to the collector again had a count rate about 10 percent of that of the detector with the collector, which is in agreement with the previous test. The count rate on the detector with the gas collector reached a maximum of about 54,000 cpm which again is in good agreement with the other tests.

In the next test, the procedure was again the same except that gas collector pads were placed on both geigers on one side of the baffle. The

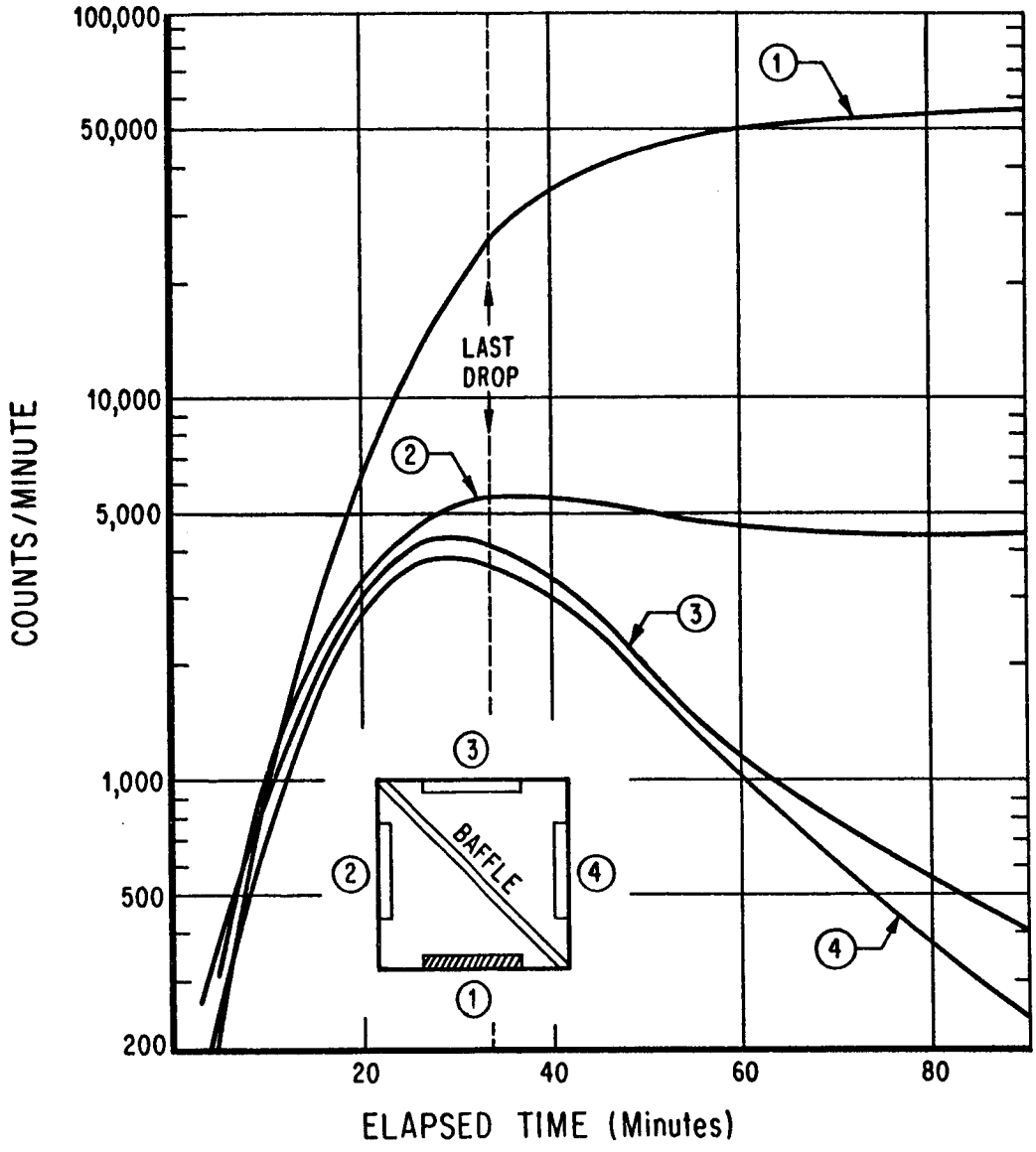


Figure IV-3. Test with One Geiger with Gas Collector and Three Bare Geigers with Plenum Chamber Divided by Baffle.

results are shown in Figure IV-4. Again, it was noticed that time required for the $C^{14}O_2$ to be reduced by a factor of two was about 10 to 15 minutes. The final count rate on the two detectors with collector pads was about 22,000 cpm and 25,000 cpm--giving a total of 47,000 cpm which is in good agreement with previous count rates for the one uc of $C^{14}O_2$ that was released.

Four gas collector pads on four geiger tubes were used as the configuration in the next test as further investigation of a possible relationship between number (i.e., area) of pads or effective detector window area and sensitivity. Figure IV-5 is the result of this run. The sum of the count rates of the four individual detectors was about 69,000 cpm. This is somewhat higher than the three previous tests, but is about equal to that obtained with the single geiger with detector mounted on the chamber as in the Gulliver III configuration. Again, it is recognized that there are variations in gas collectors and basic sensitivity of geigers which contribute to the spread in the data. A check was made to determine if these gas-collector pad-covered tubes were detecting the betas from the neighboring pads as was found with bare tubes near a pad (reference: Figure IV-2). In this configuration, there was no significant decrease in count rate when each tube and pad were removed and counted outside the chamber.

Figure IV-6 is a plot of the count rate curves from all the collector pads of Figures IV-2 through IV-5. Where multiple gas collector pads were used, the count rates of the detectors were added and the total plotted. Although it is not plotted on the figure, the data for a single geiger with gas

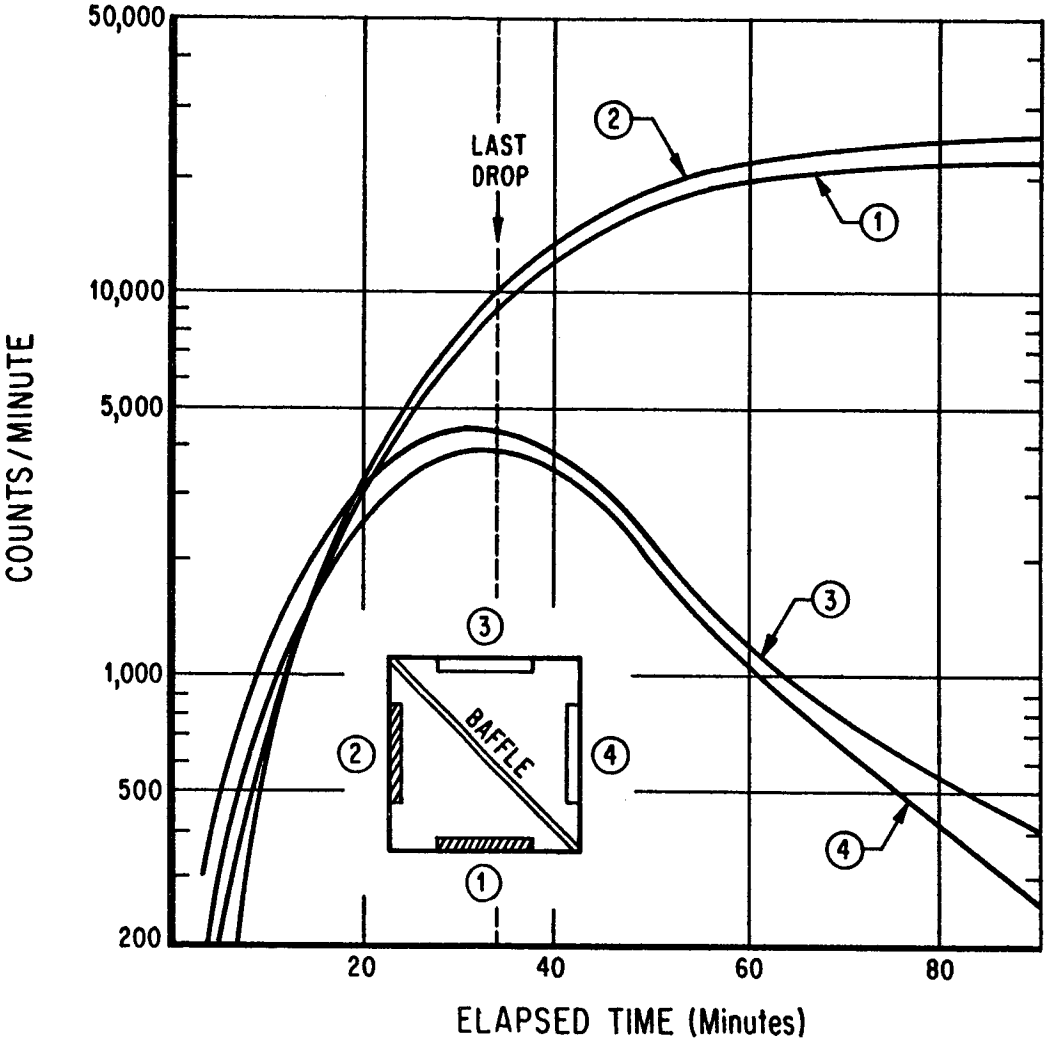


Figure IV-4. Test with Two Geigers with Gas Collectors and Two Bare Geigers with Plenum Chamber Divided by Baffle.

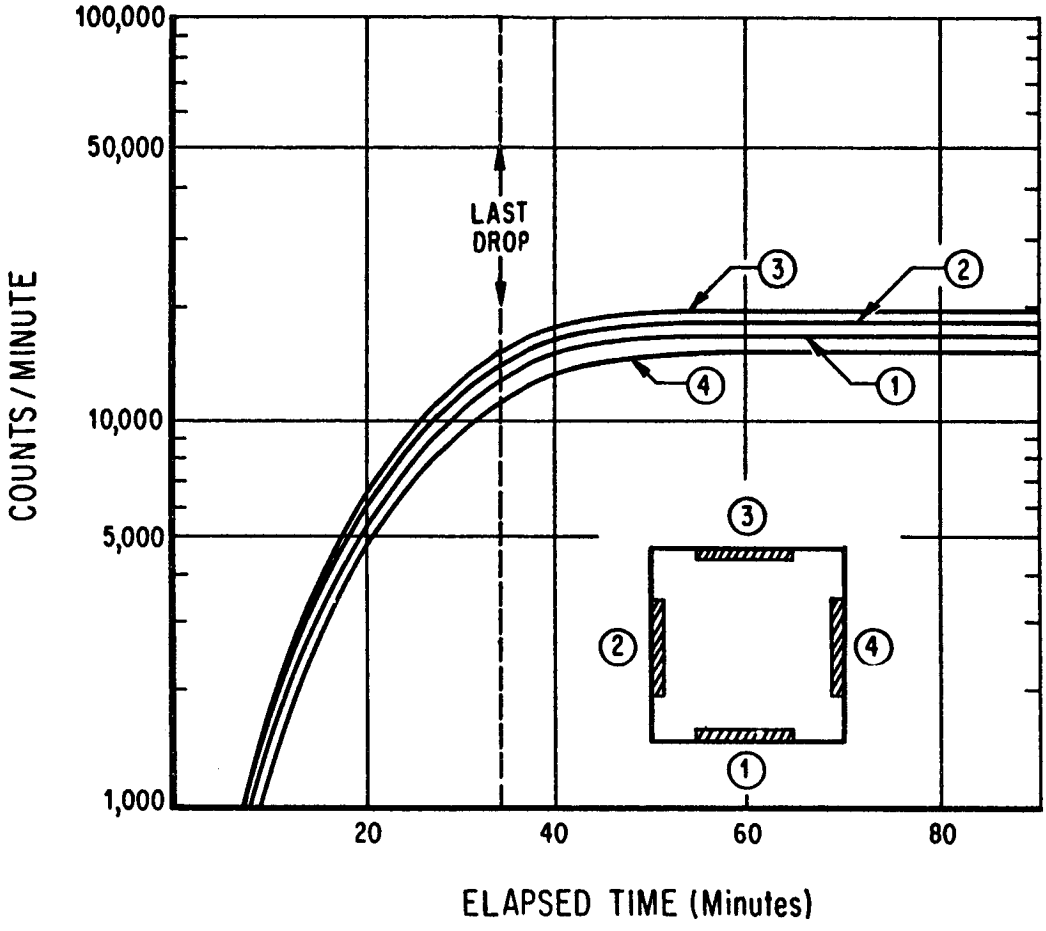


Figure IV-5. Test with Four Geigers with Gas Collectors.

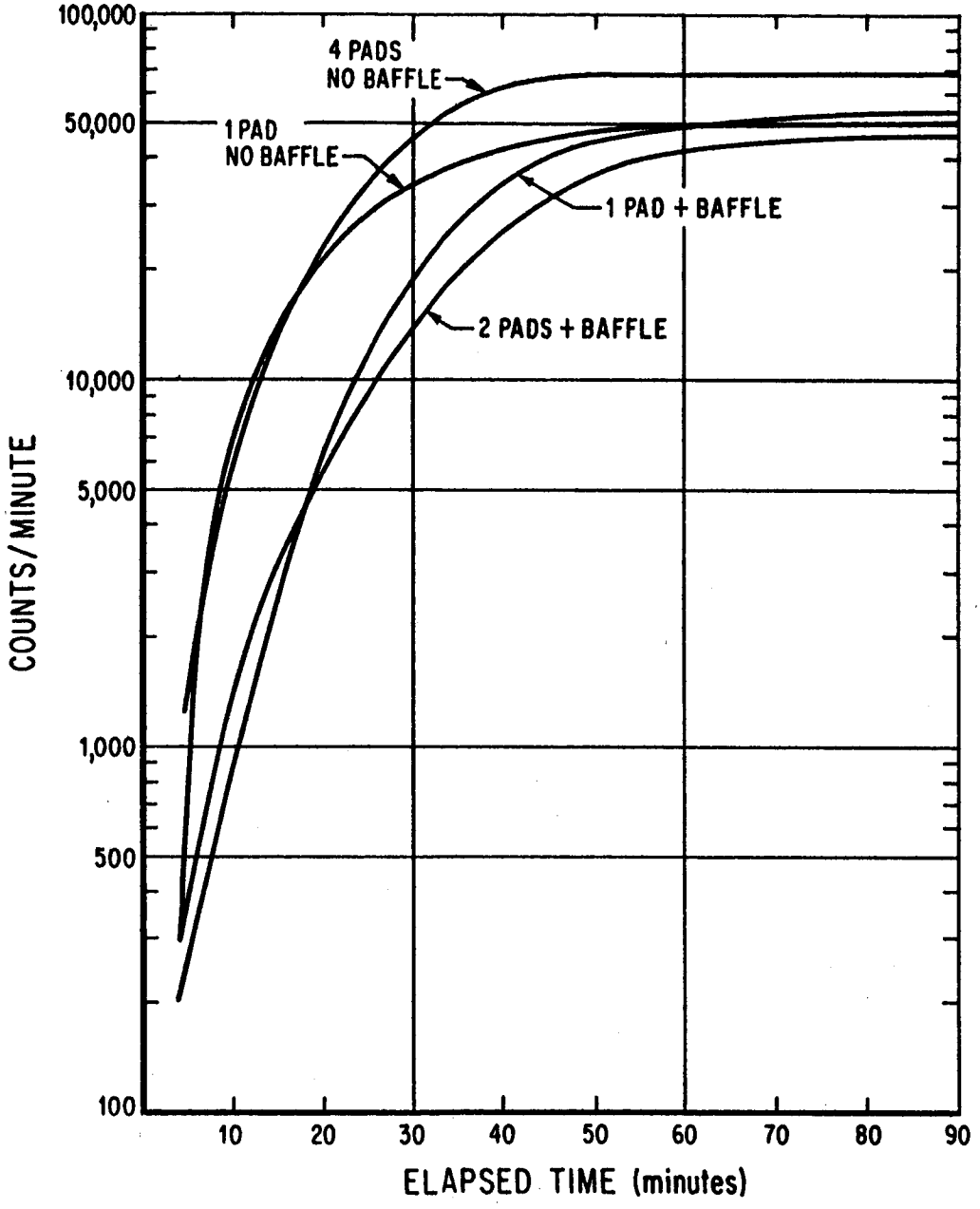


Figure IV-6. Comparison of Total Count Rates of Four Different Configurations.

collector pad in the Gulliver III configuration coincides very closely with that data for the four collector pads without a baffle. Note that these curves are in pairs--the upper are for pads without an interfering baffle, while the lower are for pads in a chamber with a baffle. In neither case is there a significant difference in sensitivity between the total count rate from multiple pads and the count rate from a single pad. Without repeating this type of test several times, no definite conclusion should be drawn, but there are strong indications that sensitivity cannot be increased significantly by increasing collector area, or detector window area, provided the quantity (moles) of CO_2 is not large with respect to the available hydroxide in the gas collector.

3. Tests with Metabolic $C^{14}O_2$

Tests that have been conducted earlier in the program have indicated that it is hazardous to draw conclusions about gas collectors based solely on results obtained by chemically generated $C^{14}O_2$. What may appear best for collecting chemically generated $C^{14}O_2$ may not be best for metabolic $C^{14}O_2$. Conclusions must await analysis of several metabolic tests. It has been speculated that the causes of these discrepancies may be saturation of collector with non-tagged CO_2 of metabolic or non-metabolic origin, degradation of collector by non-tagged metabolic products other than CO_2, or water vapor degradation of collector. The tests of this series with the detectors in the plenum chamber also showed some of these unpredictable effects.

In the first of these tests with metabolically generated CO_2, four gas collector pads were used on the detectors in the plenum chamber, and the results were compared against a single detector with gas collector mounted as in the Gulliver III configuration. The results for the incubation of identical samples of E. coli and tagged broth are shown, along with the configuration, in Figure IV-7. In comparing the sum of the count rates of the individual detectors in the plenum chamber to the count rates of the single detector on the other incubation chamber, it is evident that there is little difference for the first seven hours. Actually, for this period, the single detector appears to have a little more sensitivity. However, with additional time the sum of the four exceeds that of the single detector, attaining a factor of about four after 24 hours of incubation. The factor of four was not surprising since tests indicated the gas collectors were nearly saturated when they were checked at the termination of the test. It is difficult to draw many conclusions about the results between seven and 23 hours because data were taken with a rate meter and recorder for only one of the five detectors in the test. The broken lines on the curve indicate best guesses as to how the count rate changed between the end of work the day the test started and the rates observed the next morning with a scaler. It is to be noted that the counting rates observed after 24 hours were all in the vicinity of 100,000 cpm which is a rate at which the dead-time losses for the geigers are significant. Because the resolving time for these detectors was not experimentally measured prior

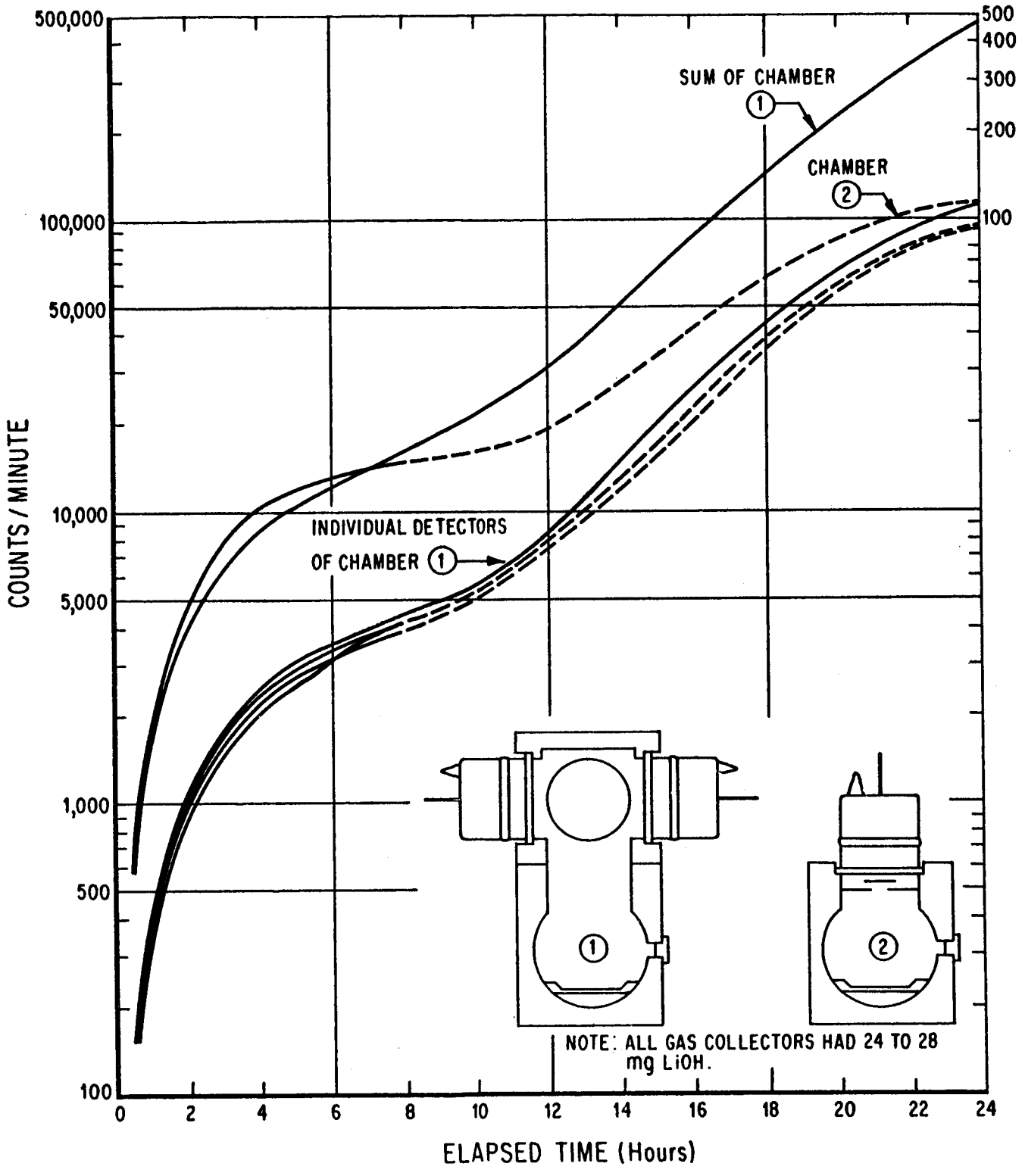


Figure IV-7. Test Comparing Metabolic $C^{14}O_2$ Detection with Four Geigers with Gas Collectors on One Unit and One Geiger with Gas Collector on Other Unit.

to the running of these tests, application of dead-time corrections was not considered worthwhile, and the data shown in the figures are uncorrected.

Three more tests were conducted with metabolically generated CO_2. In these tests four geigers without gas collector pads were used in the plenum chamber and compared to the single geiger with a collector mounted directly above the incubation chamber. The data from these tests were inconsistent among themselves. In one test the final count rate of the single detector with a gas collector was greater than the sum of the count rates of the four bare detectors; and in the other the reverse was true. Even during the first several hours of these three tests, there was no consistency. In one test, where the final sum of the four bare detectors was greater than for the single, the single detector with gas collector pad was higher for the first 17 hours; the opposite was true for the other two tests.

It is considered important that additional tests similar to those conducted with chemically generated CO_2 be conducted with metabolically generated CO_2. Parameters to be investigated in addition to those of the chemically generated CO_2 tests should be types and quantity of tagged broth and microbes.

C. TESTS WITH LARGE WINDOW GAS FLOW COUNTER

Several tests were conducted with a hemispherical gas flow counter with a window thickness about half that of the geigers used in the previous tests. The area of the window was about four to five times larger than the

geiger. This detector was mounted directly on top of the incubation chamber with the use of an adapter which permitted exposure of the entire window area to the gases emanating from the incubation chamber. The tests were conducted in the same manner as those with the multiple detectors in the plenum chamber with count rates from the flow counter being compared to the count rates from the single geiger tube mounted directly on top of the incubation chamber. Gas collectors of approximately the same thickness (mg cm^{-2}) were mounted on the face of both detectors.

In the first two tests, the $C^{14}O_2$ was generated chemically. The results of the two tests and the configuration are shown in Figure IV-8. Also shown is the ratio of the count rates of the gas flow detector to the geiger as a function of time. It is to be noticed that the quantity of $C^{14}O_2$ generated in each unit was 0.5 uc rather than the 1.0 uc which was used in the previous tests with chemically generated CO_2. The fact that the count rate of about 12,000 cpm on the single geiger was only about 1/4 that of the previous tests, rather than 1/2 as expected from the quantity of C^{14}, is somewhat puzzling. One possible explanation is that all these gas collector pads came from a different fabrication batch, but results of tests earlier in the program would not suggest that such a difference would occur.

Again, the gas collectors converted most of the $C^{14}O_2$ to carbonate shortly after the generation ceased. It is noted that the ratio of the count rates was fairly constant, showing an advantage of about 4.6 for the gas flow

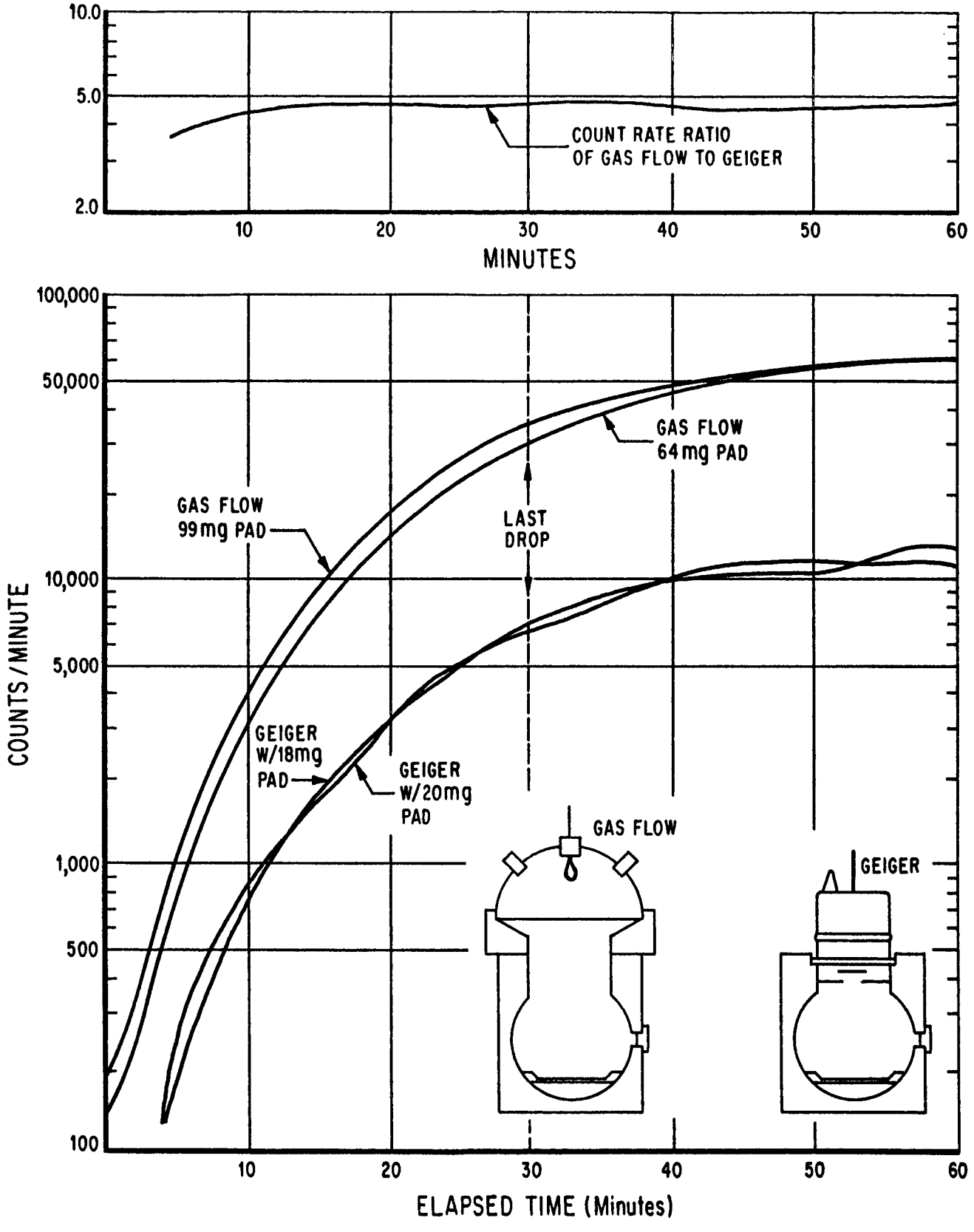


Figure IV-8. Test Comparing Chemical $C^{14}O_2$ Detection by Large Area Gas Flow Proportional Detector with Gas Collector Pad to Single Geiger with Gas Collector Pad.

unit, even before the last drop of $Na_2C^{14}O_3$ solution was deposited on the citric acid crystals.

In the next test, the results of which are shown in Figure IV-9, the $C^{14}O_2$ was generated metabolically. The ratio of the count rates of gas flow to geiger detector was again fairly constant at about 5.5 after the first hour, but was a little higher than the ratio for the chemically generated $C^{14}O_2$. Unfortunately, only one of these tests was conducted and the test ran for only eleven hours. It can only be speculated that the ratio of the count rates would have increased eventually since the gas detector on the geiger would have had less capacity than the one on the gas flow detector.

The check the basic sensitivity of these two detectors, a standard 0.11 uc C^{14} source was placed in contact with the windows and counted. The count rates were 51,700 cpm for the gas flow and 8,860 cpm for the geiger used. This gives a count rate ratio of 5.8 and over-all detection efficiencies of 21.4 percent and 3.6 percent. The source was not counted with the flow detector operating in a windowless fashion, but it is speculated that the count rate would not have been more than about 70,000 cpm for an efficiency of about 35 percent. Geometry would limit the efficiency to 50 percent unless the source (gas collector) was mounted so that both sides of the collector could be monitored.

The ratios of the count rates of the gas flow and geiger detectors for the tests are considered to be very consistent and indicative of a real

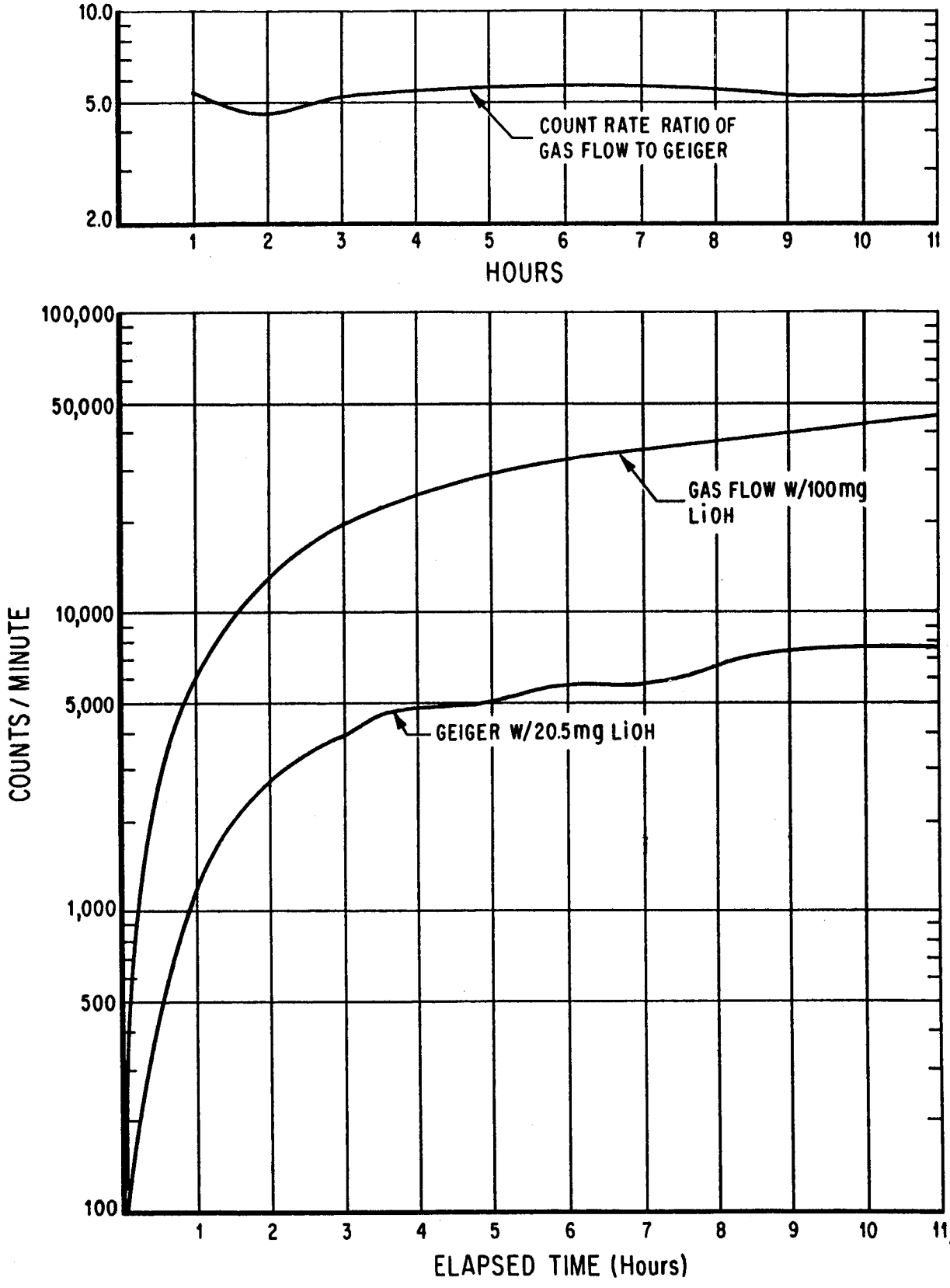


Figure IV-9. Test Comparing Metabolic $C^{14}O_2$ Detection by Large Area Gas Flow Proportional Detector with Gas Collector Pad to Single Geiger with Gas Collector Pad.

difference in sensitivity. The fact that the standard source gave the same ratio as the tests with collected CO_2 is considered excellent evidence that, for quantities of CO_2 not approaching levels which will saturate the gas collector, there is no increase in Gulliver system sensitivity to be gained by the employment of a detector that has a window any larger than the one currently employed in the Gulliver instrument.

In an attempt to explain the factor of about five in sensitivity observed between the gas flow and geiger detectors, several factors must be considered. The gas flow detector window thickness was just under 0.9 mgm/cm^2. This is compared with the window of about 1.5 to 1.7 mgm/cm^2 on the geigers used on Gulliver III. This thinner window is about equal to the maximum range of a 25 Kev beta particle; and the 1.7 mgm/cm^2 thickness is about the maximum range of a 35 Kev beta. This 10 Kev energy increment contains probably no more than 15 percent of the betas of the classical C^{14} spectrum. Since the spectrum of the C^{14} betas coming out of the gas collector would be degraded by self-absorption in the collector, the percentage in the 25 Kev to 35 Kev increment would be higher but probably not as much as 25 percent. The percentages of the betas penetrating the windows of 1.7 mg/cm^2 and 0.9 mg/cm^2 might be expected to be about 50 percent and 70 percent. This difference in transmission would result in increasing sensitivity by a factor of 1.4, which is less than the factor of about five observed. This means that basic detector sensitivity factors (such as electric

field strength and pattern and ionization potential of the detector gas) account for a factor of about 3.5 since the over-all factor would be derived from the product of the two factors, i.e., $1.4 \times 3.5 = 5$.

The anode of this particular gas flow detector is much too delicate to consider for use in a flight instrument, but other types of more rugged anodes might be utilized to permit a detector of this type to be employed.

D. ATTEMPT TO INCORPORATE GAS FLOW DETECTOR INTO GULLIVER III SYSTEM

Three special gas flow detectors were ordered from a manufacturer with anticipation of their being utilized on the two Gulliver III instruments and the uninoculated sterile unit for terrestrial field test purposes. The windows of these detectors were to be 0.00025" mylar and about 2" in diameter. The manufacturer considered it probable that these detectors could be operated in the geiger mode at a voltage less than 1000 volts with a counting gas mixture of 99.4 percent neon, 0.5 percent bromine, and 0.1 percent argon. The configuration of the anodes of this detector was considered good to provide an electric field pattern that would have good uniformity across the diameter of the tube window, and the thin window should have transmitted a larger fraction of the betas than the geigers previously employed. The larger area window would have permitted using a larger gas collector which would have improved the situation on saturation of gas collectors.

Use of such a large detector for a flight instrument would have entailed complications in providing a means to reduce the ambient Martian background radiation contributions to the detector count rate.

The basic design of this detector was for use as a sealed geiger with an 0.010" stainless steel window, and modification to use the thinner window required operation in a gas flow mode.

Testing of these detectors in the laboratory before incorporation into the Gulliver instruments was without success. The operating voltage was in the vicinity of 1500 volts and performance was extremely erratic. When it was determined that the detectors would not operate satisfactorily, they were returned to the manufacturer with an agreement for refund.

PART II. REVIEW OF PROGRAM FOR THE YEAR

A. GENERAL

1. Objectives

The objectives of the program, a subcontract from Resources Research, Inc. on Contract NASr-10, for this year were grouped into four categories in the following order of priority: (1) Maintenance of the multichannel automatic recording system used at Resources Research, Inc. in their experimental program; (2) supporting local and out-of-town field testing of the Gulliver III instruments; (3) investigation of improvement of radioisotope detection sensitivity; and (4) investigation of modification of other components of the system to improve sensitivity. During the second quarter there was an instructed re-direction of the efforts to put more emphasis on radioisotope detection, metabolic collection, non-metabolic gas removal, and soil sample collection--in that approximate order of priority. The local field testing efforts were reduced drastically and plans were made to condense the out-of-town testing.

The laboratory investigations along these objectives were intended to improve terrestrial field test response of the Gulliver system with consideration given to parameters that could be affected by environments encountered in a Mars mission; however, no efforts were to expended specifically for hardening for such a mission.

2. Personnel

This program was conducted in the Space Instrumentation Department of the American Machine & Foundry Company's Research & Development Division. The activities of this Department are under the direction of Dr. A. A. Sterk as Department Manager and Mr. David Jaffe, the Assistant Department Manager. Mr. A. Wendell Carriker was Project Manager. The main areas of endeavor and the names of other AMF personnel who worked principally on the program are as follows: David E. Winer, Engineer--gas collector, gas removal, and detectors; Joseph L. Bowles, Physicist--detectors; Richard L. Simms, Engineering Technician--field testing, gas collection and removal, detectors; and Arthur Powers, Electronics Technician--field testing, detectors.

B. FIELD TESTS

At the beginning of the program the materials utilized in field testing (such as squibs and ampules which were carried over from the previous year's program) were depleted to a level such that field testing could not be continued until supplies were replenished. As a result, the field testing effort during the first quarter was limited to three tests. During the second quarter the local field testing program moved along smoothly with no significant mechanical or electronic difficulties encountered. With this situation indicating that a reasonable degree of reliability had been attained for purposes of normal terrestrial field tests, it was judiciously

decided that the local field testing effort be curtailed in order to put more emphasis on the parameters directly related to system sensitivity, such as radioisotope detection and gas collection.

The original program plan called for field tests to be conducted at several locations around the country to be selected by Resources Research, Inc. on the basis of soil and climate conditions. Another decision made to save time and money was that several of these environmental conditions could be attained during one field trip of a more extended nature rather than on four or five separate trips. As a result, the number of out-of-town trips was reducted to two. One of these out-of-town tests was conducted in September in Orange County, Virginia, on the grounds of the Piedmont Experimental Station of the Virginia Polytechnic Institute. This site was selected because of the high iron content of the soil. All equipment performed satisfactorily for this test.

The other field test trip was to California, where "home base" operations were set up in the laboratories of Dr. Norman Horowitz at the California Institute of Technology. Field tests on this trip were conducted at Sheep Mountain in the White Mountain range at an elevation of about 12,000 feet; on sand dunes near the edge of Death Valley; and on alkaline desert near the Salton Sea in Southern California. The performance of the instrumentation during these latter three tests was satisfactory for collection of significant data. However, there more more difficulties encountered in these

three tests than had been encountered in all the other tests performed during the year.

A more detailed description on the performance of the instruments during these out-of-town field tests is contained in the Eleventh Quarterly Report.

Two significant changes have been effected in the equipment used:

First is the addition of an uninoculated sterile control unit to provide information to supplement that obtained with the two Gulliver III instruments which provide the test and antimetabolite inhibited control data. This uninoculated sterile control unit is of the same internal configuration as the Gulliver III instruments; but the functions of breaking the broth ampule, rotating the spool on which the sterile collection lines were previously wound, and opening the baffle-valve between the incubation chamber and gas collector were accomplished manually rather than by automated devices controlled by the programmer. The gas collector and radiation detector were the same as in the Gulliver III instruments, and the detector count rate was read out in the same manner. This unit was not provided with a small auxiliary tank for flushing nonmetabolic C^{14} gas products.

The second addition to the field test set-up was the incorporation of an RRI-provided count rate meter and recorder for automated collection of data from the three units utilized in the field tests. To effect this automated data recording required that minor modifications be made in the

programmer and the design and fabrication of a timer and switching system to apply sequentially the output signals from the test unit, the inhibited unit, and the uninoculated sterile unit to the input of the rate meter.

C. RADIOISOTOPE DETECTION

Throughout the course of the program the state of the art of various detectors capable of measurement of the soft betas from C^{14} has been followed by reviewing appropriate technical literature. Although advancements in the field of semiconductor detectors have been noted, it was not considered necessary to do any experimental work with such detectors within the bounds of the objectives of this program. Many of the considerations which determined it inadvisable to use scintillation detectors (described in the reports of the two previous years) still prevail, and no experimental work was done with these detectors.

The tests during this program were restricted to two detector types: gas flow proportional detectors and geiger detectors. During the first two quarters of the program, the principal efforts in the area of radioisotope detection were focused on investigation of parameters to be considered if a windowless counter were utilized in the Gulliver system. The two most obvious possibilities for a windowless detector are: (1) an ionization chamber, and (2) a detector with gas amplification--that is, one which operates in the proportional or geiger mode.

Several factors work against consideration of the ionization chamber. To operate in the ionization mode, there must be sufficient ionization energy deposited in the detector volume to provide currents that are measurable. Considering the anticipated quantities of metabolic $C^{14}O_2$ that would be present early in the incubation period, the currents which would be available for measurement from an ionization chamber of dimensions of any practical size (for example, the volume of the present total instrument) would be less than 10^{-15} amps. Measurement of such currents with any degree of accuracy would require a major development effort for a Mars mission instrument. The current would be even lower when the low atmospheric pressure of Mars is also considered because the ion pairs formed per centimeter beta particle path length in the ion chamber would be even less than on Earth.

For a windowless counter with gas amplification, the gas for the detector presents significant difficulties. One possibility is to operate the incubation chamber and the detector in the same gas environment--the gas being either the normal gases of the atmosphere or a gas optimized for detector performance (such as a mixture of argon and methane or helium and butane). To design a detector using an atmospheric gas of unknown composition would be a difficult task, if not an impossible one. To use a common gas mixture such as argon and methane is not considered to be a very good approach from the biological point of view in that the microbes in the incubation chamber would be subjected to a gaseous environment considerably different from their natural habitat.

Another possibility for a windowless counter with gas amplification would be to utilize a detector which is alternately separated from the incubation chamber when it is filled with a counting gas during a counting mode and then intimately exposed to the gases of the incubation chamber after having been flushed with atmospheric gases at the end of the counting mode. This type of detector system is feasible but would entail paying a severe penalty in size, weight, power, and complexity if it were incorporated into a Mars mission instrument.

In the first two quarters of the program, laboratory tests were conducted in an attempt to determine the maximum gain in sensitivity which might be attained if it were possible for a windowless counter to be employed, and to determine the effects of various parameters on the performance of such a detector. As expected, it was determined that water vapor is a severe problem in terms of operation of a detector using a conventional counting gas, namely argon and methane. Tests described in the Tenth and Eleventh Quarterly Reports indicate that very significant increases in sensitivity can be attained for metabolically generated and chemically generated $C^{14}O_2$ provided the water vapor is eliminated. Some of these apparent factors of increased sensitivity over the single thin-window geiger tube with a gas collector mounted in the window were in excess of a factor of ten. Much of these data were subject to question because it was determined during this same period of time through other tests that there were major differences

in sensitivity of gas collectors utilized in these detector tests. When it was decided late in the second quarter that it was an untenable situation to try to use a gas amplification type detector with an unknown gas and, similarly, it was untenable to expose the microbes to a foreign gas, investigations of this type stopped.

The only other windowless detector tests were performed with a mock-up system which permitted alternating the gas in the detector. The results obtained with these tests (three) indicate that the factor of sensitivity over that of a single thin-window geiger tube (such as employed in Gulliver III) was about a factor of four to six. No conclusion should be drawn from this small number of tests; but the previously mentioned penalties of size, weight, power, and complexity made it inadvisable to test further.

A number of very significant tests were performed during the final quarter of the program. These are described extensively in the first part of this report. These tests gave rather conclusive evidence that, contrary to results of earlier testing, there is little to be gained in system sensitivity by employing detectors which will accommodate larger area gas collectors than those that are currently used on the Gulliver III instruments, provided the quantity of CO_2 is less than the capacity of the collector. Tests were performed which permitted calculations to be made to determine sensitivity for a thin inner wall, hollow cylindrical detector with multiple anodes for counting only gas phase CO_2 relative to the sensitivity of a single geiger

with a gas collector. The contribution of gas phase CO_2 to the total count rate of a geiger detector when a gas collector is used was determined to be insignificant. The rate at which the CO_2 gas is converted to carbonate by an LiOH gas collector was determined--the time for half the CO_2 in the chamber to be converted to carbonate was about 10 to 15 minutes. The increase in sensitivity which appears to be attainable with measurements made with a thin window proportional counter which has a very uniform internal electric field was found to be about the same as that which was attainable with the windowless flow counter alternately filled with counting gas and the gas from the incubation chamber. Additional tests are sorely needed to establish unequivocably parametric information indicated by some of the tests of the last quarter.

D. GAS COLLECTION

1. Krylon Versus Cellulose Gum as Collector Adhesive

At the beginning of this contract year, the method of applying gas collector to the Gulliver III detector was by spraying the geiger tube window with Krylon and then spraying the collector substance on before the Krylon dried. On drying, the Krylon acted as an adhesive. The method had the disadvantages of not allowing uniform coatings, not providing means for reproducing weight of collector, and leaving the weight of Krylon an unknown factor. Cellulose gum was tried as an adhesive because it seemed to give better sensitivity, it could be applied evenly, and it was possible by mixing

a given proportion in the collector to determine the amount of each substance. The experiments which showed that the gum was a promising adhesive were made using chemically generated $C^{14}O_2$. But, when a series of runs was made using metabolic $C^{14}O_2$, the superior sensitivity of gum as shown in the "chemical" experiments disappeared, and in fact the old Krylon adhesive was superior. Because Gulliver III procedures must be compatible with metabolic evolution of gas, the gum adhesive was dropped from further consideration.

2. Sensitivity and Reproducibility of Gas Collectors

Experiments were performed which indicated that the combination of Krylon plus barium hydroxide exhibited only fair reproducibility. Several approaches were tried in a search for a novel and, hopefully, superior means of applying collector. The best of these was production of wafers of hydroxide impregnated with figerglass tissue in which the exact weight of each substance was known, and in which the hydroxide crystals were uniformly distributed in the fiberglass tissue. The fiberglass tissue which appeared to be most suitable for this process was an AMF product known as Tissuglas 200-G.

It was found that pads of proper size could be cut from a sheet of impregnated Tissuglas and stored in air-tight containers without affecting their sensitivity. Further, the reproducibility of barium hydroxide pads

prepared by this method was such that equal weight pads produced count rates upon exposure to a given quantity of tagged CO_2 within 20 percent of each other.

Sensitivity experiments with various combinations of Tissuglas thickness and solution strength showed, as expected, that the thinner materials were more sensitive to small quantities of C^{14} tagged CO_2; but that thicker materials, with more hydroxide and greater absorptive capacity, would convert a larger quantity of CO_2 to carbonate and therefore appeared to be more favorable for situations where the quantity of CO_2, natural or tagged, was large. The sensitivity of the barium hydroxide pad is similar to the Krylon-barium hydroxide combination.

3. Lithium Hydroxide as Gas Collector

The melting point for barium hydroxide is 78°C, a temperature well below the 135°C sterilization temperature to be applied to the Mars spacecraft. A brief investigation of this point was made by placing barium hydroxide pads in an oven at temperatures beyond the handbook melting point of the compound, and by heating the powder gently over a flame. It did not melt in the oven, but did so in the more severe heating of the flame. Lithium hydroxide was chosen as a substitute because of higher melting point and greater capacity for CO_2 per unit weight. Sensitivity and reproducibility comparisons were made between the Li and Ba compounds. LiOH appeared

more sensitive to metabolic CO_2, but about equal to $Ba(OH)_2$ when chemical CO_2 was used.

Reproducibility of the LiOH pads was not consistent enough, so the technique of making them was further refined. Results have been reasonably satisfactory in this respect since then. The method of fabrication is described in the Eleventh Quarterly Report.

The effect of exposure to the atmosphere on LiOH pads was investigated by fabricating a large quantity under CO_2-free conditions, and then exposing batches of these to the same quantity of tagged CO_2 but after varying exposure time to the atmosphere. No drastic effect was noted even for exposure as long as five hours, but it should be mentioned that the data spread was greater than is desirable for making firm conclusions.

4. Effect of Gas Collector Pad Area on Sensitivity

This subject is discussed in more detail earlier in this report. The conclusion expressed there is that no gain in sensitivity results from increasing the area of collecting pad, provided the quantity of gas does not cause saturation.

E. NONMETABOLIC GAS REMOVAL

Nonmetabolic tagged gas in solution in the broth causes the sterile control level of Gulliver III to be high if this gas is not removed before the baffle-valve is opened between the incubation chamber and the detector.

Flushing provisions are included in the Gulliver III design. Laboratory experiments have indicated that breaking the broth ampule and flushing with reasonable quantities (100 ml) of gas at atmospheric pressure is not as effective as breaking the ampule at the reduced pressure of Mars (considered 0.1 earth atmosphere at the time) and allowing expansion and fast diffusion out of the incubation chamber via the string port.

In an experiment performed by breaking an ampule of untagged broth saturated with $C^{14}O_2$ and counting the gas phase CO_2 above the baffle in the incubation chamber, it was found that 15 minutes of diffusion, without flushing, at 0.1 atmosphere would eliminate nearly all the CO_2 from a dummy incubation chamber. This test was not as sensitive to traces of gas as in Gulliver because no collector was used and only gas phase C^{14} was counted. However, such tests provide information on the rate that the $C^{14}O_2$ leaves the incubation chamber.

More sensitive tests were conducted using collectors on the geiger tubes, again in 0.1 atmosphere, but not opening the baffle until after 15 minutes of diffusion. In these tests, Mars atmospheric gas composition and pressure were also simulated by a constant flow through of a gas mixture (97 percent N_2 and 3 percent CO_2). The results showed that, indeed, a quantity of tagged CO_2 remained to be collected after the diffusion period, although the bulk of the gas had been eliminated.

The next experiment was to learn the relative amount of this residual nonmetabolic gas when standard Gulliver III sterile control runs were

made--one in the earth atmospheric pressure and the other in the simulated Mars conditions. From this one run it appeared that the effect of diffusion to Mars atmosphere as compared to earth atmosphere was one of significant (almost a factor of ten) reduction of the sterile control level. No comparison test was conducted to compare sterile control levels of a unit flushed, as is done during field tests, to a unit exposed to the reduced pressure.

The recent indications that the Mars atmospheric pressure is even lower than that supposed for these experiments leads to the conclusion that if such lower pressure exists it will be beneficial from the standpoint of increasing system sensitivity because lowering the sterile control level improves the signal-to-noise ratio of metabolic activity (signal) and nonmetabolic sterile control activity (noise).

APPENDIX A

SEQUENCE OF OPERATIONS FOR FIELD TEST

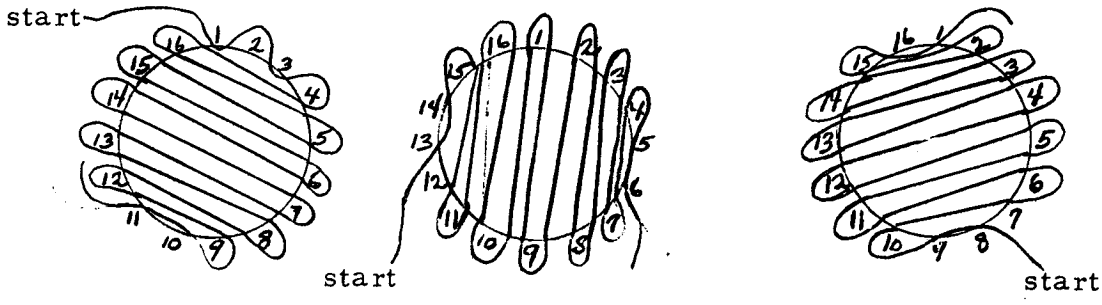
I. MECHANICAL

A. Gas Collectors

1. Fabricate LiOH on Tissuglas collector material (technique described in Eleventh Quarterly Report).
2. Cut collector to size and weigh.
3. Store in container containing Ascarite.

B. Projectiles

1. Wash, rinse, and dry string and chenille.
2. Cut to proper lengths.
3. Tie string and chenille together.
4. Grease string and chenille with silicone grease.
5. Wind lines on jig starting with chenille, as shown in diagram below.



1st Layer 2nd Layer 3rd Layer

6. Using pencil eraser or plug the same diameter as the inner diameter of jig, press winding tight after every layer or two.
7. Feed string through opening in back of projectile, put projectile over jig, extrude winding into projectile, and put cap on projectile.

C. Load and Seal Antimetabolite Ampules.

D. Flushing Tanks

1. Install bellows-type squib in plug.
2. Check operation of tank valve and integrity of seal.
3. Pressurize tank to about 78 psi with air nozzle packed with sterile cotton.
4. Install plug with bellows squib.

E. Instrument Body

1. Lubricate "O" rings, bearing, and pins.
2. Cut and install filter paper around spool.
3. Secure leader-lines to spool.
4. Install gaskets and insulation on squib leads.
5. Cut and install sponge near baffle.
6. Check operation of retrieval motor manually.
7. Assemble as far as possible without installing ampules, according to isometric drawing and other drawings.
8. Tie string from projectiles onto leaders and secure projectiles to instrument with rubber bands.
9. Wrap instrument and tools in aluminum foil and autoclave.
10. Using foil as a sterile field and wearing sterile gloves, complete assembly.
11. Mount gas collector in detector assembly.
12. Mount electronic assembly on mechanical unit.
13. Mount instrument in base of nose cone.
14. Complete wiring to terminal board on nose cone base.

F. Field

1. Insert projectile driver rods and projectiles in guns
2. Put nose cone over base and assure opening line-up with guns.
3. Manually actuate uninoculated sterile control unit.

II. ELECTRONICS

A. Pre-assembly

1. Check voltage plateau and pulse shape of geigers
2. Using ice and soldering iron, check operation of thermostat.

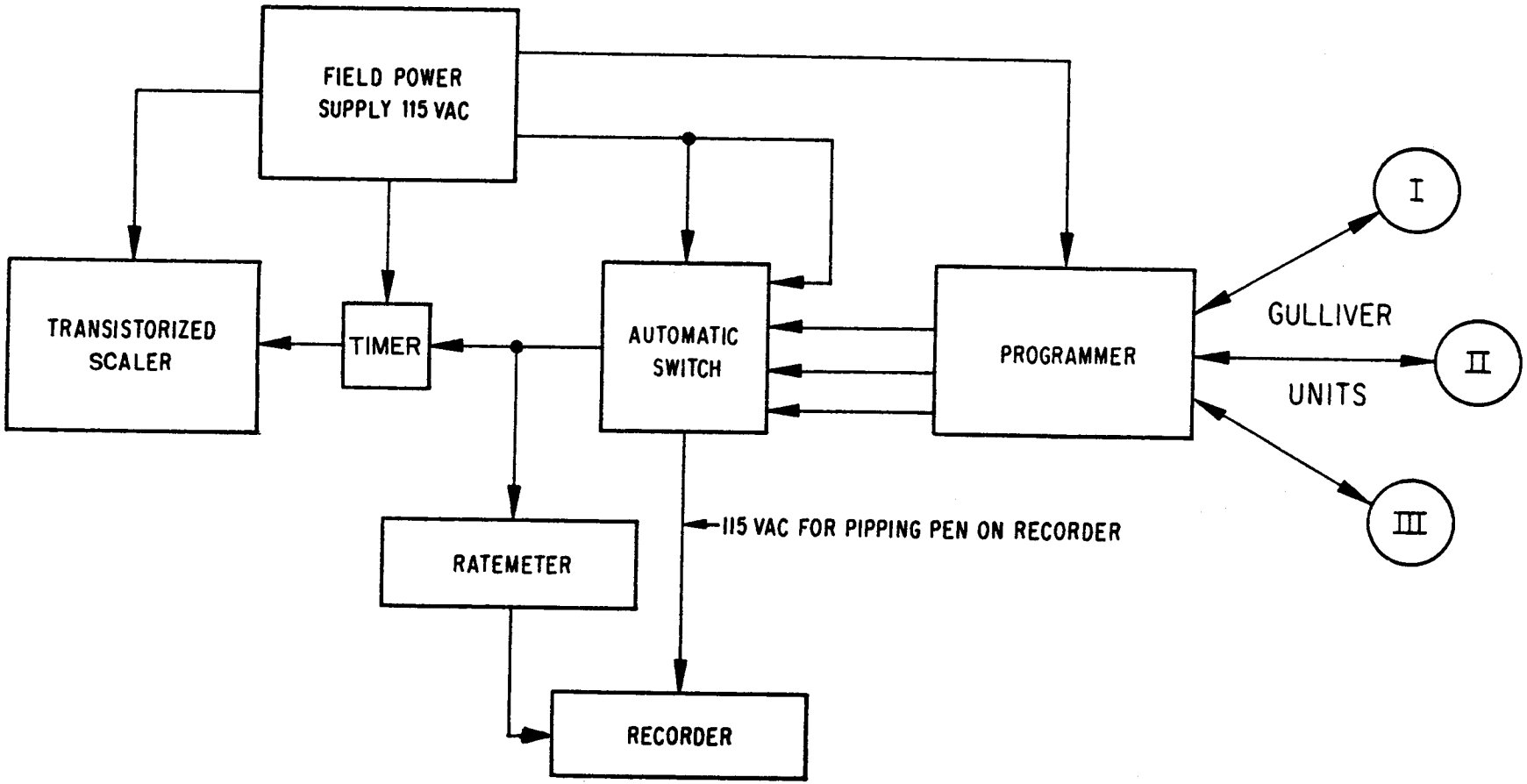
3. With electronics assembly plugged into programmer and field power supply with the aid of an oscilloscope:
   a. Check pulse shape, frequency, and amplitude of motor drive circuit
   b. Check width of anticoincidence pulse using reference source
   c. Check pulse shape and rate of beta detector pulses using a reference source
4. With programmer plugged into field power supply and with jig with lights for each programmer channel plugged into programmer, run programmer through cycle and check time and sequence of programmer signals.
5. Charge storage batteries and check condition of cells just before going into the field.
6. Check the 28 volts d.c. in the programmer.

B. Assembly

1. With instrument mounted in nose cone base, check condition of squibs with galvanometer-type squib tester.
2. Complete wiring according to diagram.
3. Check wiring according to diagram.
4. Check counting rates of background and reference source for all three units.

C. Field

1. Assure that all switches are in proper position with special attention to antimetabolite switch guard being down.
2. Assure that programmer cams are in the "start" position.
3. With projectiles in guns, actuate motors to take in excess string.
4. Hook up according to block diagram below.
5. Take background count rates on all three units.
6. Assure that heaters and thermostats are working properly.
7. Start cycle with programmer.
8. Take count rates with manual switching of outputs of units into transistorized scaler.
9. After antimetabolite is injected into control unit, clip the antimetabolite injector leads of other unit.
10. Periodically check output voltage of field power supply as data are being recorded.



BLOCK DIAGRAM OF FIELD TEST EQUIPMENT